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CD5, A MOLECULAR SWITCH REGULATING SIGNALING AT THE SURFACE OF T CELLS

CD5, UM REGULADOR MOLECULAR DA SINALIZAÇÃO DAS CÉLULAS T

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Doutor Alexandre do Carmo, Investigador Principal, e da Professora Margarida Fardilha, Professora Auxiliar Convidada da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro.

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“The only way to do great work is to love what you do (…)’

Steve Jobs

o júri

presidente

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S100A4 regulates the Src-tyrosine kinase dependent differentiation of Th17 cells in rheumatoid arthritis.
Biochimica et Biophysica Acta. In press.

No poster apresentado no congresso internacional *EMBO Lymphocyte Signalling*:

Rita F. Santos, Ana Mafalda Santos, Jairo Pascual, Martina Bamberger, Georges Bismuth, Simon J. Davis, Alexandre M. Carmo.
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palavras-chave

Ativação dos linfócitos T; CD5; fosforilação de tirosinas; recetor inibitório; vias de ativação intracelulares; complexo de sinalização; Crk; S100A4.

resumo

O reconhecimento por parte do receptor de células T (TCR) de péptidos processados e apresentados pelas células apresentadoras de antigénios (APC) via MHC envolve a formação de uma zona de contacto célula-célula, chamada sinapse imunológica. Após ativação das células T, diversas vias de sinalização celular são ativadas culminando na indução de transcrição génica. A glicoproteína de superfície, CD5, é considerada um inibidor da ativação via TCR, sendo uma das primeiras moléculas a ser recrutada para a sinapse imunológica após ativação da célula T. No entanto, até ao momento nenhum ligando na célula apresentadora de antigénios foi devidamente identificado e aceite como sendo o ligando do CD5. No nosso grupo, foi estabelecido que a sequência da cauda citoplasmática do CD5 que contém dois resíduos de tirosina (Y429 e Y441) é determinante para a sua função inibitória bem como para a translocação desta molécula para a sinapse imunológica. Neste projeto, propomos dissecar o papel de cada uma destas duas tirosinas nas propriedades modulatórias do CD5. Com esse propósito, foram introduzidas substituições na molécula do CD5, substituindo cada uma das referidas tirosinas por fenilalaninas. Estes mutantes foram expressos numa linha celular negativa para a expressão de CD5 endógeno e ajudarão a desvendar os mecanismos moleculares induzidos após ativação das células T e através dos quais o CD5 exerce o seu papel inibitório, avaliando por exemplo, fosforilação intracelular, fluxos de cálcio, e proliferação. Os nossos resultados nos ensaios de cálcio e de proliferação vieram confirmar o papel inibitório do pseudo-ITAM e, mais precisamente, da tirosina Y429. Estes mutantes serão também analisados por microscopia de fluorescência para avaliar a sua responsabilidade na mobilização do CD5 para a sinapse imunológica. Adicionalmente, será também monitorizada esta mobilização num mutante desprovido da porção extracelular, de forma a determinar a necessidade de estimulação por parte de um ligando expresso na célula apresentadora de antigénios. Um dos nossos resultados pioneiros mostram uma associação entre o CD5 e a proteína adaptadora Crk, que poderá funcionar como uma ponte para outras interações. A proteína dependente de cálcio S100A4 também se associa com CD5 bem como a Fyn e Lck, levando-nos a propor que S100A4 controla os mecanismos de proliferação e de diferenciação de populações de células T auxiliares, dependentes de Lck e de Fyn, respetivamente, através de um mecanismo dependente do CD5. No futuro, CD5 poderá ser usado como alvo terapêutico com o objetivo de modular a resposta imune.

keywords

T cell activation; CD5; tyrosine phosphorylation; inhibitory receptor; intracellular pathways; signalosome; Crk; S100A4.

abstract

T cell receptor recognition of peptide-MHC expressed on antigen presenting cells (APC) involves the formation of a tight cell-cell contact area, named immunological synapse. Upon successful T cell activation, several signal transduction pathways are triggered culminating with the induction of gene transcription. The T cell surface glycoprotein CD5, an inhibitor of TCR signaling, is one of the molecules that targets to the immunological synapse upon T cell activation, although no ligand in the APCs has yet been discovered. We have determined that a sequence containing two tyrosine residues (Y429 and Y441) within the cytoplasmic domain of CD5 is determinant both in the inhibitory function as well as in the synaptic localization of the molecule. In the current project we dissect the role of each of the two tyrosine residues in the modulatory properties of CD5. For this purpose, tyrosine-to-phenylalanine substitutions were introduced in the CD5 molecule, which was expressed in a CD5-negative T cell line. These mutants will be relevant for unveiling the molecular mechanisms induced upon T cell receptor-mediating triggering, such as intracellular phosphorylation, calcium signals and proliferation. Our results in calcium and proliferation assays confirm the inhibitory role of the ITAM sequence, and more precisely, of the Y429 residue, although the other tyrosine may also play a somewhat lesser part. These mutants will also be analyzed by fluorescence microscopy for their ability to translocate to the immunological synapse upon T cell interaction with superantigen-loaded APCs. An additional CD5 mutant, one devoid of the extracellular domain, will also be monitored and will determine the requirement for an APC-expressed ligand to induce CD5 translocation to the synapse. We found two new putative intracellular binding partners that can be part of, and help assembling, CD5 signalosomes. *In vitro* studies show that CD5 can associate with the adapter protein Crk, maybe acting as a bridge to other protein-protein interactions. On the other hand, the calcium-binding protein S100A4 also associates with CD5, Fyn and Lck. We hypothesized that S100A4 controls Lck-dependent T-cell proliferation and Fyn-dependent differentiation of T helper subsets through a CD5-dependent mechanism, highlighting once again a putative role of CD5 as a molecular switch. In the future, CD5 can be used as a therapeutic target in order to modulate immune responses.

Contents

Figures

ABBREVIATIONS

APC - Antigen-presenting cells

ATP - adenosine 5'-triphosphate

BCR - B cell receptor

CD - Cluster of differentiation

CFSE - carboxyfluoresceinsuccinimidyl ester

CK2 - Casein kinase 2

Crk - CT10 Regulator of Kinase

Csk - C-terminal Src kinase

c-SMAC - Central supramolecular activation complex

CTLA-4 - Cytotoxic T lymphocyte associated protein-4

DAG - Diacylglycerol

d-SMAC - Distal-SMAC

FACS - Fluorescently-activated Cell Sorting and Analysis

Grb2 - Growth factor receptor-bound protein 2

ICAM-1 - Intercellular adhesion molecule 1

Ig - Immunoglobulin

IL - interleukin

$InsP_3$ - inositol-1,4,5-trisphosphate

IP - Immunoprecipitation

IS - Immunological synapse

ITAM - Immunoreceptor tyrosine-based activation motif

ITIM - Immunoreceptor tyrosine-based inhibitory motif

Itk - IL-2-inducible T cell kinase

JTAg–JurkatTAg cells

LAT - Linker for activation of T cells

Lck - Lymphocyte-specific tyrosine kinase

LFA-1 - Lymphocyte function-associated antigen 1

mAb - Monoclonal antibodies

MAPK - Mitogen-activated protein kinase

MHC - Major histocompatibility complex

NK - Natural killer

NKT - Natural killer T cells

NMMII - Nonmuscle myosin II

NMR - Nuclear magnetic resonance

PAG - Glycosphingolipid-enriched microdomains

PAMP- Pathogen-associated molecular pattern

PFA - paraformaldehyde

PHA - phytohemagglutinin

PI - propidium iodide

PI3K - Phosphatidylinositol 3-kinase

PKC - Protein kinase C

PLC-γ1 - Phospholipase C-γ1

pMHC - Peptide/MHC

PRR - Pattern-recognition receptor

p-SMAC - Peripheral supramolecular activation complex

PtdIns(4,5)P_2 - Phosphatidylinositol-4,5-bisphosphate

RasGAP - RasGTPase activating protein

SH2 - Src-homology 2

SHP-1 - SH2 domain-containing protein tyrosine phosphatase 1

SLP-76 - SH2 domain-containing leukocyte protein of 76kDa

SRCR-SF - Scavenger Receptor Cysteine-Rich Superfamily

Tc - T cytotoxic

TCR - T cell receptor

Th -T helper

ZAP-70 - ζ-chain associated protein with 70kDa

INTRODUCTION

1. OVERVIEW OF THE IMMUNE SYSTEM

The tissues, cells and molecules responsible for immunity constitute the immune system, which is in constant alert in order to protect our organism against the billions of microorganisms such as bacteria, viruses, parasites or fungi that we are constantly exposed to [1]. A remarkable feature of the immune system is the capacity to distinguish between self and non-self, and to neutralize pathogens without damaging own tissues [1].

There are two broadly defined types of immune responses in mammals that are complementary and partly overlapping [2]. The first and early line of defense is provided by the innate arm of the immune system (phylogenetically the oldest defense system) [1]. This is characteristically very fast and consists of cellular and biochemical defense mechanisms that are in place even before any threat appears, and is poised to respond rapidly to infections [1]. This type of immunity is composed of physical and chemical barriers, phagocytic and natural killer (NK) cells, blood proteins and cytokines, all regulating many cellular activities of innate immunity [1].

The innate system is not antigen-specific; however, it is able to discriminate foreign molecules from self [2]. Phagocytes bear pattern-recognition receptors (PRRs) that identify structures termed pathogen-associated molecular patterns (PAMPs) present in microbe-derived molecules but not in host cells [3]. PAMPs are usually molecules essential for the microorganisms to survive and therefore unlikely to be altered [3], such as components of fungal and bacterial cell-walls, β-glucan, peptidoglycans and lipopolysaccharides [4]. PRRs have the advantage of being constitutively expressed in the host and of having the ability to detect pathogens at any given life-cycle stage. Specific PAMPs can be recognized by different PRRs, which activate particular signaling pathways [4].

In addition to this first line of defense, a new type of response has emerged throughout evolution in vertebrates, the adaptive immune response. This type of response is characterized by very specific and more complex responses and is based on specialized antigen-specific groups of cells, the B and T lymphocytes [1]. Each T or B lymphocyte (also known as T or B cells) expresses at their surface unique receptors that are specific to one given antigenic determinant, *i.e.*, T cell receptors (TCR) or B cell receptors (BCR) expressed on individual cells are different from those of nearly all other T or B cells in the same animal and recognize different antigens. On the other hand, the adaptive response develops a memory that makes

future responses against the pathogen more quick and efficient [1]. Firstly, antigens derived from the killing and phagocyte-intracellular processing of microbes are presented to and recognized by the antigen specific T or B cells, leading to cell priming, activation, differentiation and proliferation, which normally occurs in secondary lymphoid tissues. Secondly, the effector response against the pathogens takes place in the sites of disease/infection [1, 5]. After the termination of the response, some of the expanded T and B cells remain in the system as memory cells, ensuring a fast response once and if the same pathogen re-infects the host later on in life [1].

It is currently accepted that the innate and adaptive immune systems act together [2]. In an initial phase, pathogens are identified, uptake and processed by specialized phagocytic cells such as dendritic cells or macrophages, and antigenic peptides are loaded into major histocompatibility complex (MHC) molecules and exposed at the cell surface to be presented for T and B cell recognition. Innate pathogen sensing and phagocytosis regulate cytokine production, MHC peptide presentation and co-stimulatory molecule expression in these so-called antigen-presenting cells (APC). From the T lymphocyte side, recognition of a specific peptide presented in the context of an MHC complex of the APC leads to an efficient activation of naïve T cells. Furthermore, the antigen-specific cells amplify their responses by promoting innate effector mechanisms and recruiting innate immune cells to bring about the complete control of the invading microbes [5, 6].

An illustration of how thin, and sometimes inexistent, is the separation between the innate and adaptive responses is exemplified by natural killer T cells (NKT) and γδ T cells, which are implicated in both types of immunity. NKT cells identify and neutralize potential pathogens without previous activation, being capable of detecting and responding even in the absence of expression of MHC molecules by the APC, thereby eliminating the cellular target and producing cytokines that give rise to other mechanisms of the immune response [7,8]. The γδ T cells, which express a different form of TCR, mediate NK cell cytotoxicity [9] and phagocytosis [10].

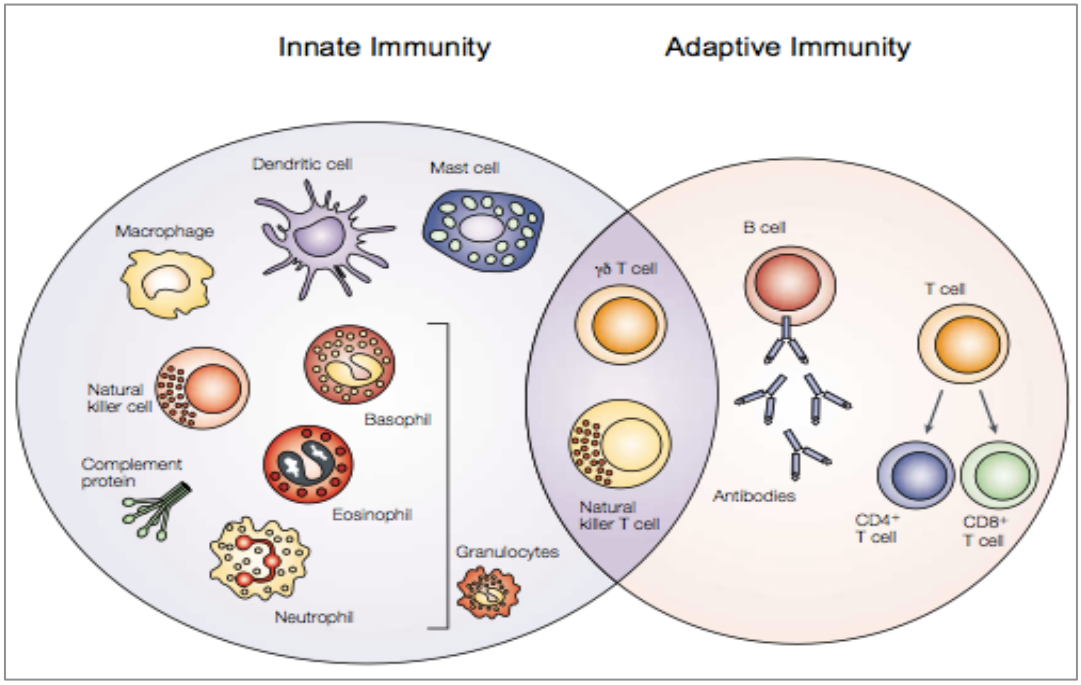

Figure 1. Collaboration between innate and adaptive immunity. The immune system has two broadly defined types of immune response that work together, leading to complexes and efficient responses. The innate response, as a first line of defence, is faster and is activated through complement proteins, soluble factors and different effector cells, like macrophages, dendritic cells, granulocytes, mast cells and natural killers. As a second, and more complex response, emerged the adaptive arm of immune system, which introduced specificity and memory, with T and B cells as mainly intervenients. However these two types of response connect in some points. NKT and γδ T cells established the bridge between the innate and adaptive immune system. (Image from Dranoff, 2004).

2. T LYMPHOCYTES

T lymphocytes differentiate from progenitor cells in the bone marrow and then migrate to the thymus to mature. There, only T cells that do not react with self-antigens presented by thymic epithelial cells or tissue macrophages are able to leave the thymus as mature naïve T cells [1].

Two major types of effector T cells have been identified, T helper (Th) and T cytotoxic (Tc), bearing mutually exclusive CD4 or CD8 co-receptors on their surface, respectively. $CD4^+$ Th cells are the orchestrating cells of the immune response, recognizing foreign antigens, producing cytokines and activating other parts of the cell-mediated immune response to eliminate the pathogen. They also play a major part in the activation of B cells. $CD8^+$ cytotoxic cells are involved in antiviral and anti-tumor activity [5].

2.1. The major histocompatibility complex

The TCR only recognizes peptides processed and presented in the context of the MHC. There are two main types of MHC molecules, class I MHC and class II MHC that present protein antigens according to their origin [1].

Antigens that are produced endogenously within the cell, like viral or tumor proteins, are processed into small peptides, transported into the endoplasmic reticulum and picked up by class I MHC molecules [11, 12]. Upon presentation of antigen derived peptides, the immune system has the indication that these cells have been infected and need to be eliminated by cytotoxic $CD8^+$ T cells, whose TCRs specifically recognize peptides in the context of this class of MHC [13].

Alternatively, specialized professional APCs (such as dendritic cells, macrophages and B cells) might have taken up exogenous antigens by endocytosis. Peptide fragments from the antigen enter in a specialized endosomal compartment thereby loading MHC class-II molecules and these peptide-MHC (pMHC) class II complexes are presented to helper $CD4^+$ T cells [14].

In summary, as a result of their different function, MHC class-I is present in all nucleated cells and MHC class-II is only expressed by professional APCs [15].

2.2. The TCR/CD3 complex

T lymphocytes express at their surface a TCR, a clonotypic heterodimer responsible for the recognition of specific non-self antigens. Each TCR chain is a member of the immunoglobulin (Ig) superfamily, possessing one N- terminal variable and one constant Ig-like domain, followed by a connecting peptide, a transmembrane domain and a short cytoplasmic tail at the C-terminus. The TCR heterodimer consists of an α and a β chain (or γ and δ in a small subset of T cells) covalently bound by disulfide bridges [16], and this receptor recognizes only processed antigens that are presented at the surface of APCs as peptide fragments of the original antigen (γδ T cells do not recognize antigen in the peptide form [17]).

The TCR is found non-covalently bound to the transmembrane CD3 signaling complex, consisting of CD3γ, CD3δ, CD3ε and CD3ζ chains, (to form CD3δε and CD3γε heterodimers and the disulfide-linked ζζ homodimer) which activate the T cell after antigen contact [18]. The presence of an antigen for the TCR-complex activation is necessary and the expression of all the components of the complex is indispensable for its assembly and recognition. The short TCR cytoplasmic tail has

no catalytic activity but the CD3 chains contain in their intracellular region specific sequences that are responsible for the initiation of the signaling cascade [1].

These regions are called immunoreceptor tyrosine-based activation motifs (ITAMs) and share a consensus sequence containing two important tyrosine residues [19]. Each CD3 γ, δ or ε chain contains one ITAM whereas ζ chains have three ITAMs per chain. ITAMs are phosphorylated by the Src family kinase lymphocyte-specific tyrosine kinase (Lck) that is tightly associated with either the CD4 or CD8 co-receptors, and each doubly-phosphorylated ITAM becomes a docking site for the ζ-chain associated protein of 70 kDa (ZAP-70), a protein tyrosine kinase containing two Src-homology 2 (SH2) domains [20, 21].

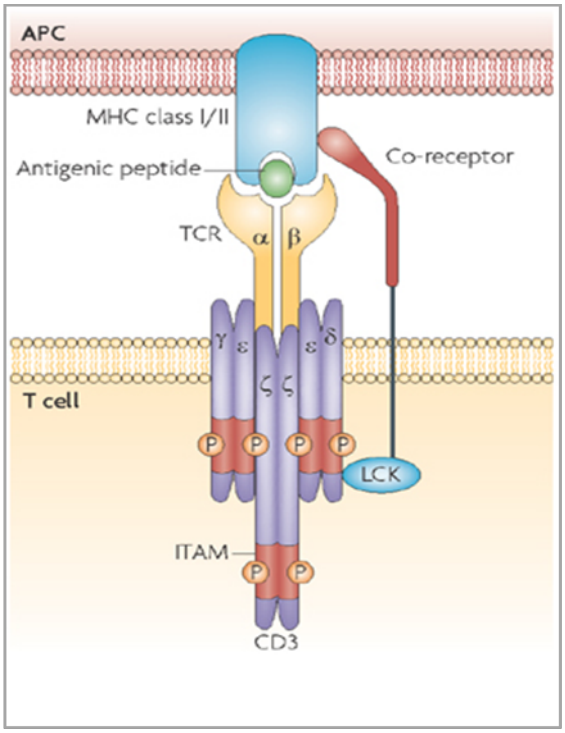

Figure 2. The TCR/CD3 complex. The T-cell receptor is a cell surface heterodimer consisting of an α and a β chain covalently bound by disulfide bridges. The TCR only recognize processed peptides presented by an APC in the context of MHC. This interaction is stabilized by the bound of the kinase Lck in the proximity of the TCR–CD3 complex. Lck can then phosphorylate the CD3-associated immunoreceptor tyrosine-based activation motifs (ITAMs). (Image from Gascoigne, 2008).

Triggering of the T cell receptor and ZAP-70 docking initiate a signaling cascade that results in the partial activation of the T cell. Several signal transduction pathways that involve protein kinases and phosphatases, second messengers and key intermediates, are then triggered, contributing to the induction of gene transcription according to the defined genetic programs that are characteristic of the different T cell subsets, and to the differentiation and proliferation of these cells [16].

2.3. T cell-APC interactions

After capturing a protein antigen in epithelia and tissues, APCs transport these antigens to draining lymph nodes where they wait for the arrival of circulating naïve T cells having TCRs specific for the displayed MHC-peptide complex [13]. Conversely, T cells continuously circulate throughout the peripheral lymphoid

organs, searching for APCs that present a foreign peptide for which their TCR is specific [22].

The first interaction between the APC and the T cell is mediated by adhesion molecules expressed in both cells that allow a physical contact, allowing the T cell to scan the APC in order to recognize the peptide presented by the MHC [13].

The recognition of the pMHC by the TCR is very specific but not enough to induce complete activation of the T cell. It can even, under some conditions, lead to T cell anergy. Costimulatory signals are thus essential for the full activation of the T cell [23, 24]. The signals generated by TCR recognition of pMHC, together with signals derived from the interaction of the T cell costimulatory CD28 with its APC-expressed ligands CD80 or CD86 (also designated as B7.1 and B7.2 respectively) will induce the complete activation of the T lymphocyte [25, 26].

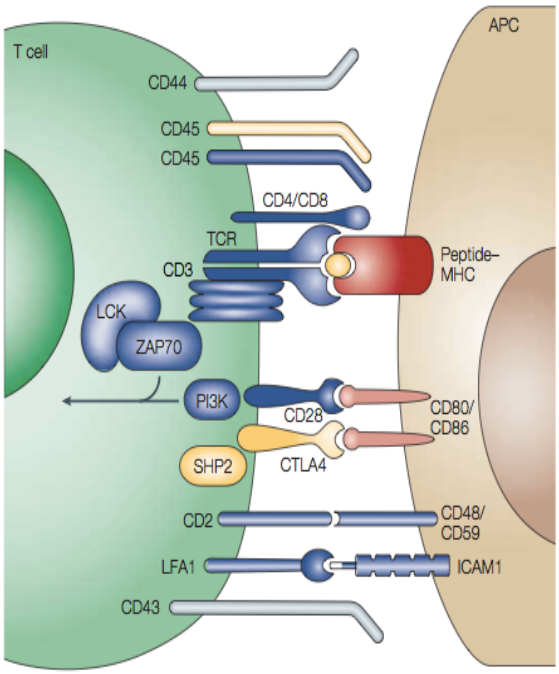

Figure 3. Key signaling molecules that are involved in T-cell recognition. The stimulatory peptide-MHC molecule is shown in red, establishing a ligation with TCR/CD3 complex. The activatory/costimulatory molecules are represented: on blue, by the other hand, inhibitory molecules are shown in yellow. There are other important molecules that influence the type of response without contributing to signaling, represented in grey. (Image adapted from Huppa and Davis, 2003).

Upon phosphorylation of specific tyrosine residues of the intracellular part of CD28, additional signaling proteins are recruited, and these signals are the beginning of the co-stimulatory cascade [11]. This pathway begins when CD28 interacts with its counter receptor and, despite lacking enzymatic activity by itself, CD28 can be phosphorylated also by Src-family kinases, resulting in an increase of the association between CD28 and Phosphatidylinositol 3-kinase (PI3K), IL-2-inducible T cell kinase (Itk) and Growth factor receptor-bound protein 2 (Grb2) [27]. Later, with the assembly of this net of interactions, cytosolic calcium increases, the Mitogen-activated protein kinase (MAPK) signaling pathway is initiated and specific transcription factors are activated, which will determine the fate of the T lymphocyte [28, 29].

CD28 has a homolog that exerts an inhibitory effect during T cell activation. This homolog is the cytotoxic T lymphocyte associated protein-4 (CTLA-4) [30]. The

competition between CTLA-4 and CD28 for the same two ligands (CD80/CD86) together with the superior affinity of CTLA-4, results in the replacement of CD28 binding and consequently down modulates T cell activation [31].

Apart from the co-receptors (CD4 or CD8) and costimulators (CD28) or repressors (*e.g.*, CTLA-4), a number of other essential molecules will influence the type of response, such as CD2, Lymphocyte function-associated antigen 1 (LFA-1), CD44 and CD45, among others [16].

2.4. The immunological synapse and T cell signal transduction

Upon recognition of peptide-MHC complexes by the TCR, a stable cell-cell interface is formed between the T cell and the APC involving an extensive rearrangement of the plasma membranes of both cells, and is named the immunological synapse (IS) [32]. To maintain this stable architecture, diverse structural changes are required that lead to a dynamic action of the actin cytoskeleton and the activation of adhesion receptors in these areas [32, 33].

The first studies using fixed-cell imaging revealed that the IS is organized in a "bull's eye" pattern [33, 34] with a central cluster containing TCR-pMHC pairs. This area is termed the central supramolecular activation complex (c-SMAC) and is the place where coreceptors (CD4 and CD8), the costimulatory receptor CD28 and small low-affinity adhesion receptors such as the CD2-CD58 pair, can be found [33, 35]. This region is surrounded by a peripheral supramolecular activation complex (p-SMAC), a ring mainly composed by integrins like the LFA-1 and, on the APC side, its ligand intercellular adhesion molecule 1 (ICAM-1) [33]. The large CD45 glycoprotein shows a particular behavior, it is recruited to the c-SMAC at an early time point, possibly to activate Lck, and later on moves to a more peripheral region than the p-SMAC. This farther region is termed distal-SMAC (d-SMAC), being the place where most other large molecules such as CD43 are localized [36].

These first studies also implied that TCR signaling was initiated and sustained at the c-SMAC. However, this view has been gradually changing, and supported by several mechanistic and structural studies, currently it is thought that T cells can be activated in the absence of c-SMAC formation [33].

Just within a few minutes upon TCR triggering and TCR/CD3 complex activation, there occurs a distribution of the different T cell surface receptors and an extensive membrane reorganization, driven by actin remodeling and polymerization

processes [37]. The central role of actin involves its attachment to proteins and positioning them into the center of the IS [38].

TCR microclusters formed immediately after the recognition of pMHC were described as responsible for the initiation and sustaining of TCR signaling, being sufficient to increase intracellular concentration of calcium [39, 40].

However, the exact mechanism of selective TCR triggering remains unclear and many models have been proposed. According to the molecular mechanism these models can be divided into aggregation [41], conformational change [42] and kinetic segregation [43]. These models are explained in more detail in Figure 4 [44].

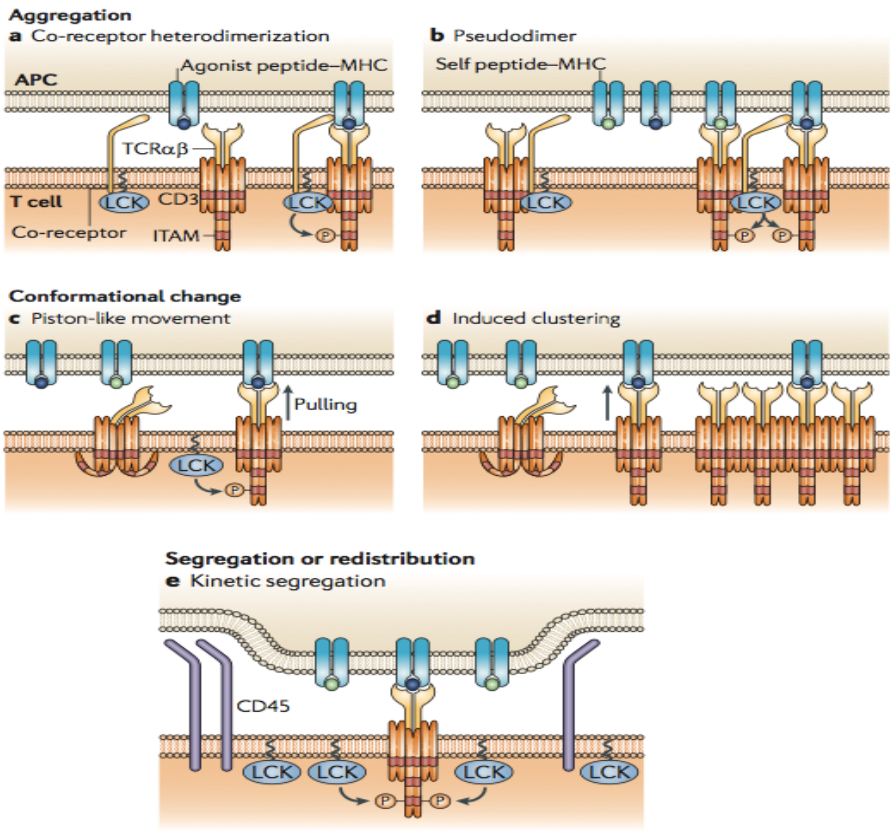

Figure 4. TCR triggering models. A and B. Aggregation models. In the co-receptor heterodimerization model (A), a co-receptor is responsible for allowing Lck to be in close proximity with the TCR/CD3 ITAMs by binding the MHC. In the pseudodimer model (B), a dimer is formed when a self-pMHC and an agonist pMHC bind adjacent TCRs, and the co-receptor brought by the TCR-self-pMHC pair engages the agonist pMHC. **C and D.** Conformational change models. A piston-like conformation (C) is adopted by the TCR/CD3 complex upon mechanical effects of pMHC binding, leading to a conformational change in the CD3 cytoplasmic domains. The induced clustering model (D) allows an increase in kinase activity targeting the TCR/CD3 ITAMs. **E.** The kinetic segregation model postulates that inhibitory tyrosine phosphatases are segregated from the close-contact zone formed between T cells and APCs upon TCR binding to pMHC, enhancing phosphorylation of the TCR/CD3 ITAMs. (Image from van der Merwe and Dushek, 2011).

Once T cell activation is initiated, the TCR signaling pathway (Fig.5 [45]) begins with the recruitment of Lck, which promotes the phosphorylation of Fyn, and both induce the phosphorylation of TCR/CD3 ITAMs [20]. After these initial steps, ZAP-70 is recruited to the doubly-phosphorylated ITAM tyrosines, and is in turn phosphorylated and activated [46]. Activation of ZAP-70 results in the phosphorylation of linker for activation of T cells (LAT), which works as signaling docking platform for phospholipase C-γ1 (PLC-γ1), PI3K and SH2 domain-containing leukocyte protein of 76kDa (SLP-76) [47, 48].

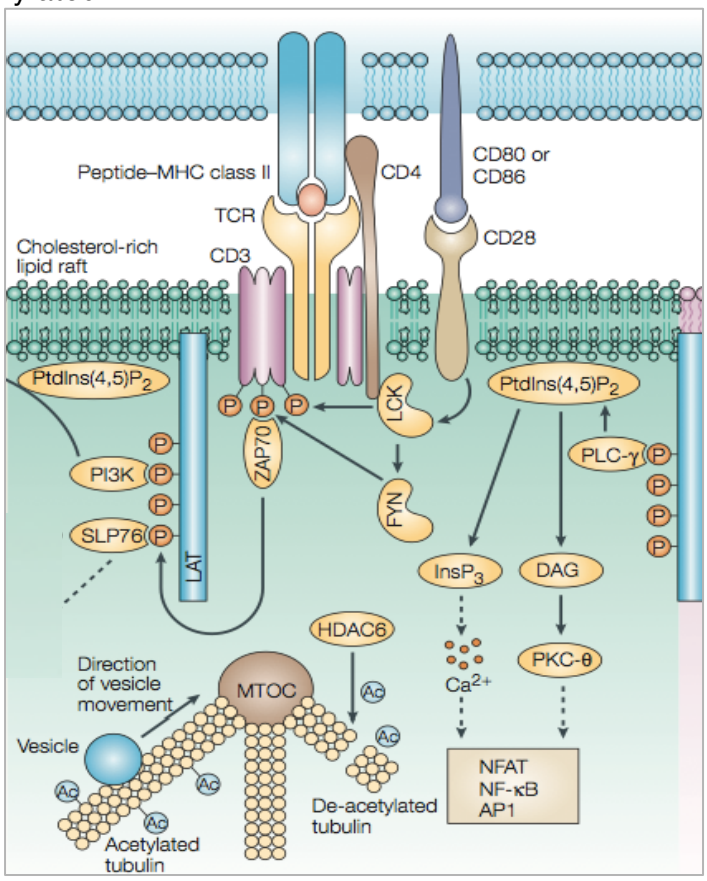

Figure 5. Schematic representation of the T cell signal transduction. Upon TCR triggering and the engagement of costimulatory receptors, Lck promotes Fyn phosphorylation and both induce the phosphorylation of CD3 ITAMs. This leads to the recruitment of ZAP-70 and consequently to the phosphorylation of LAT. PLC-γ1, PI3K and SLP-76 docks to LAT and recruit/activate different effectors. Membrane phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) releases diacylglycerol (DAG) and inositol-1,4,5-trisphosphate ($InsP_3$) that induce calcium release. These events activate downstream transcription factors such as NF-κB, NFAT and AP1. (Image adapted from Friedl et al., 2005).

Later, with the assembly of this network of interactions, several signal transduction pathways that involve protein kinases and phosphatases, second messengers and key intermediates, are triggered, cytosolic calcium increases and the MAPK signaling pathway is initiated. All together these events culminate in the induction of transcription factors that are translocated to the nucleus, leading to differentiation, proliferation and actin reorganization responses, which will determine the fate of the T lymphocyte [20, 29, 49].

This system is tightly regulated and mechanistic failures or imbalances may lead to severe consequences such as autoimmunity, allergies, and chronic infections, these being some of the reasons why the regulation of T cell activation mechanism has being a highly investigated topic [50].

3. THE INHIBITORY SURFACE GLYCOPROTEIN CD5

CD5 is a T cell surface glycoprotein that represses tyrosine phosphorylation-mediated signal transduction pathways and modulates cellular activation. Its expression is restricted to thymocytes, mature peripheral T cells and also to a subset of mature B cells (B-1 a cells) [51]. CD5 was also one of the first surface markers used to identify T cells due to it early appearance in thymocyte development [52].

CD5 is a type I transmembrane glycoprotein with a molecular mass of 67kDa and belongs to the highly conserved scavenger receptor cysteine-rich superfamily (SRCR-SF) [53]. Structurally, CD5 has an extracellular region with 348 amino acids organized in three SRCR domains, an hydrophobic transmembrane sequence of 29 amino acids, and a 94 amino acid-long cytoplasmic tail [53]. The cytoplasmic domain of CD5 is highly conserved and contains multiple serine and threonine residues and four tyrosine residues (at positions Y378, Y429, Y441 and Y463). Of these, three are potentially embedded into known tyrosine-based phosphorylation motifs [54]. The amino acid sequence surrounding Y429 and Y441 resembles an ITAM, differing in only one residue from the ITAM consensus sequence [55]. On the other hand, residues Y378 and Y441F are often considered to be part of an immunoreceptor tyrosine-based inhibitory motif (ITIM) [56].

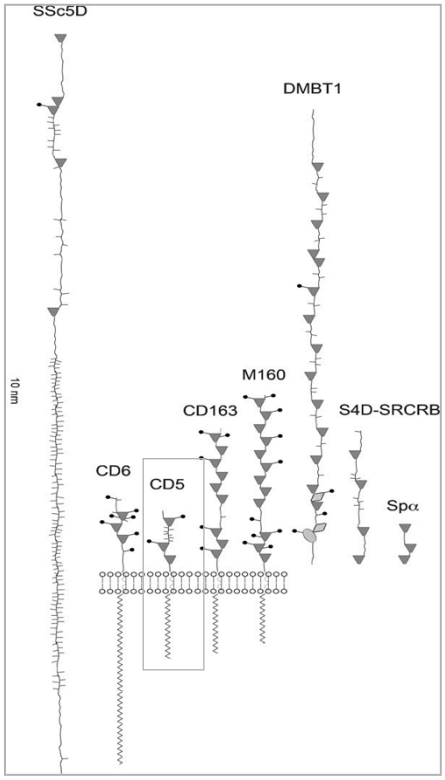

Figure 6. SRCR-SF group B members. CD5 with three SRCR domains (represented as grey trapezoid) in the extracellular part, a transmembrane region and a cytoplasmic tail. (Image adapted from Gonçalves et al, 2009).

Upon TCR triggering, residues Y429 and Y463 are phosphorylated with very rapid kinetics [57-59], with the protein tyrosine kinase Lck (the kinase that coincidently phosphorylates the TCR/CD3 ITAM motifs) playing a major role in these phosphorylation events[59-61]. Two other protein tyrosine kinases, Fyn and Itk, may additionally contribute to CD5 phosphorylation or even regulate this Lck function[48].

Several candidates for being CD5 ligand have been described. The first reported ligand was a C-type lectin expressed in B cells, CD72 [62]. Other APC potential ligands were described later, such as gp40-80 [63], gp150 [64]and IgV(H) framework sequence region [65]. However, since the interactions of these proteins with CD5 were never independently confirmed, the CD5 ligand remains unknown. Recently, CD5 has been show to establish homotypic interactions [66].

From a biological and functional point of view, CD5 was initially proposed to behave as a costimulator [67] but later was found to be in fact an inhibitory molecule [68]. The first *in vitro* studies indicated that CD5 was capable of enhancing TCR-mediated cell proliferation in peripheral T cells, working as a costimulatory molecule [67]. However the generation of a CD5 knockout mouse uncovered its inhibitory role, with the mice revealing a hyperresponsive phenotype to thymocytes and peripheral T cells. In parallel with the increase in proliferation, mice with a disrupted CD5 gene exhibited enhanced phosphorylation of signaling effectors such as LAT, PLC-γ1 or Vav, in response to TCR stimulation [68, 69].

The integrity of the cytoplasmic domain of CD5 was shown to be crucial for its inhibitory role during thymocyte development [52]. Also, the levels of CD5 expression on T cells nearly correlate with the avidity of antigen-specific receptors on T cells being important during T cell development [70]. B cells can also modulate CD5 expression by switching the use of exon 1 to alternate between a CD5 membrane-bound form and a CD5 cytoplasmic-expressed form [71].

3.1. Phosphorylation and binding partners for CD5

The inhibitory role of CD5 was first described to result from the docking of SH2 domain-containing protein tyrosine phosphatase 1 (SHP-1) (phosphatase with two SH2 domains and with an important inhibitory role in TCR signaling [72]) to the phosphorylated residue Y378 of CD5 [56]. This association was reported in the Jurkat T cell line, in murine thymocytes and also in B-1a cells [56,73,74], but the fact that CD5 mutants that do not contain this residue still retain the capacity of inhibiting signaling [75, 76] led to the conclusion at the time that SHP-1 was not the only mediator of the inhibitory effects of CD5.

At the surface of T cells, CD5 also associates with CD2, which may enhance the inhibitory function of CD5 by the enhancement of the SHP-1 activity [73, 76]. However, other molecules were independently described to associate with the cytoplasmic tail of CD5, potentially conferring additional negative regulatory

activities, such as RasGTPase activating protein (RasGAP), Cbl, Akt and Casein kinase 2 (CK2) [55, 77-79]. The CD5 tyrosine residues within ITIMs are Y378 and Y441, although curiously it has been pointed out that it is the residue Y429, which harbors these molecules that down-modulate cellular activation in thymocytes, such as RasGAP and Cbl [55, 80, 81].

CD5 signaling additionally activates a pathway that involves PI3-kinase, with the two SH2 domains of PI3-kinase binding to phosphorylated Y441 and Y463, and depending on the signaling environment, this association can also promote inhibitory effects [55, 82].

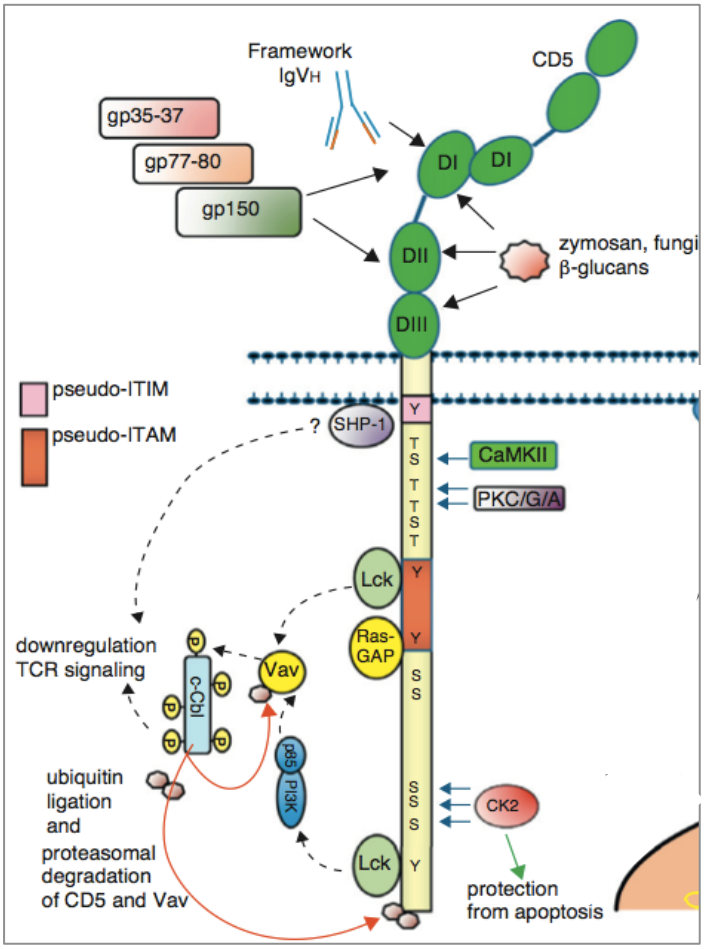

Figure 7. CD5-mediated interactions with endogenous and/or exogenous ligands. The extracellular part of CD5 establishes interactions with the described ligands and with itself (homotypic interaction). In the cytoplasmic tail, pseudo-ITAM is shaded in orange. Intracellular signaling molecules with activation roles that interact with the CD5 cytoplasmic tail are: Lck, PI3K; with inhibitory roles: c-Cbl, Ras-GAP, SHP-1; and a prosurvival molecule: CK2. (Image adapted from Soldevila et al, 2011).

As described above, Lck is the kinase responsible for the phosphorylation of CD5 at residues Y429 and Y463. It has been long known that CD5 is rapidly phosphorylated upon TCR stimulation [83], but since the TCR/CD3 complex does not include Lck, this described interaction between CD5 and Lck could be promoted in assembled signaling micro-domains, such as lipid rafts [60]. Therefore, receptors like CD5 may act as scaffolds leading to the interaction between distinct types of rafts and their components [84]. In such a role, we have observed that upon activation, CD5 associated with the Src-type kinase Fyn in lipid rafts [60].

Phosphorylation of the C-terminal Y531 residue of Fyn leads to a considerable reduction in its kinase activity [85]. This effect can be obtained through the activation of CD5 and involves the distal region of the cytoplasmic domain of CD5. Since C-terminal Src kinase (Csk) is the only kinase described to phosphorylate Fyn at this

C-terminal inhibitory tyrosine residue, we anticipated that CD5 could associate with Csk. However, we were not able to detect such an association between CD5 and Csk. One other possibility was that the CD5-Csk interaction was indirect and might be mediated by the membrane-bound adapter phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), which binds the distal part of the CD5 cytoplasmic tail. Interestingly, these associations, while promoting Fyn's Y531 phosphorylation and decreasing Fyn activity, also induced the disruption of a signaling loop involving Fyn, Csk and PAG. All this set of complex events contributed to confer yet another additional alternative role in signaling inhibition to CD5 [60].

In summary, it seems that CD5 may inhibit early TCR signaling via several pathways. CD5 may associate with SHP-1, which by inhibiting ZAP-70 activity, directly promotes itsdephosphorylation[86, 87]. In parallel, CD5 may inhibit Fyn, which is also important for the activation of ZAP-70 [60].

3.2. CD5 at the Immunological Synapse

CD5 is one of the molecules that accumulates at the IS during antigen recognition [88]. During the formation of the IS, CD5 co-localizes with TCR/CD3 complexes and is able to refrain T cell signaling without decreasing T-cell/APC adhesion or impairing IS formation. The inhibitory effect has been assigned to the ITAM-like motif of CD5 and is augmented by the increase in the level of CD5 expression at the membrane. However, in these studies it was not described whether CD5 translocation to the synapse was due to the binding of a CD5-ligand expressed on the APC or due to CD5 intracellular interactions within the T cell [89]. Recent observations from our group suggested that when the possible interactions with unknown ligands are disrupted, CD5 is still able to translocate to the IS and this movement is dependent on specific key motifs of its cytoplasmic tail. Another study evidenced that CD5 can also be rapidly recruited to the contact zone in an Ag-independent manner [89], uncovering a possible function for CD5 in early T cell activation even before the complete formation of the IS [37].

4. CRK, AN ADAPTER PROTEIN IN IMMUNE RESPONSES

Adapter proteins are important players in most signaling pathways, acting as scaffolds making the assembly of large protein complexes. Adapter proteins do not have catalytic activity.

The adapter molecule CT10 Regulator of Kinase (Crk) belongs to a protein family that binds to several tyrosine-phosphorylated proteins through SH2-phosphotyrosine interactions [90]. Crk adapter proteins include three family members: CrkI and CrkII arise from the alternatively spliced Crk gene, and CrkL is encoded in the Crk-like gene [91]. CrkI lacks the regulatory phosphorylation site and the SH3 domain in the C-terminal, although CrkII and CrkL contain one SH2 domain and two SH3 domains (in the N- and C-termini) [92], however the functions of the different domains still remain controversial.

In T lymphocytes, Crk has been reported to form multiprotein complexes through TCR stimulation. One of the most described interaction in T cells is with Cbl. Upon TCR stimulation, Cbl becomes tyrosine-phosphorylated and associates with Crk and this association is involved in the negative regulation of the TCR [93, 94]. Cbl is a ligase that catalyzes protein ubiquitynation (a process of substrates degradation by the proteasome) of signaling effectors and their subsequent degradation in the proteasome complex, therefore acting as a negative regulator of T cell activation [95, 96]. However the exact mechanism of how the phosphorylated Crk regulates TCR signaling remains unclear. Crk also exerts some important roles in T cell cytokine signaling [97], in B cell and NK-cells regulation [98,99].

5. S100A4, A CYTOPLASMIC CALCIUM-BINDING PROTEIN

Appropriate T-cell responses and the strength of TCR-pMHC interactions are connected to cytoskeleton reorganization, where filamentous actin and nonmuscle myosin II (NMMII) play a major part [100, 101]. Activation of the TCR recruits effector molecules, with the kinases Lck and Fyn playing a central role in transducing TCR/CD3-mediated signals by phosphorylating ITAMs, activating the tyrosine kinase ZAP-70 [102], and recruiting cytoskeletal remodeling factors of the Rho GTPase family to the inner leaflet of the cell membrane. Rho GTPases are coupled to actin and NMMII, and define the duration of TCR activation [100, 101]. Suppression of the NMMII

function abolishes formation of TCR clusters at the outer edge of the T cell [103] and reduces the activity of Src-tyrosine kinases in T cells [104].

S100A4 is a small Ca^{2+}-binding protein known for its metastasis-promoting properties, but it is required for normal cell-to-cell interactions and cell motility. The function of S100A4 relies on its interactions with the cytoskeletal proteins NMMII, F-actin, and tropomyosin. Binding of S100A4 to these proteins occurs in a Ca^{2+}-dependent manner and inhibits the actin-regulated ATPase activity of myosin II [105, 106]. The disassembly of myosin filaments occurring as a result of S100A4 binding has a major impact on cytoskeletal rearrangements, cell polarization, shape changes, and motility [105, 107, 108].

The interaction between S100A4 and NMMII, beta-liprin or and the cytoplasmic domain of FcγRIIIA, occurs within protein kinase C (PKC) or CK2 sensitive regions of the target proteins [105, 106, 108]. On the other hand, the cytoplasmic tail of CD5 contains a PKC-sensitive region at S427 and a CK2 phosphorylation site at its C-terminal region[109]. A deletion of the C-terminal fragment of CD5 containing the CK2-phosphorylation site results in the failure to produce the Th2 and Th17-cell subsets [109], and moreover, CD5 knock-out mice have a very similar phenotype to that presented by mice with a disrupted S100A4 gene, suggesting a functional coupling between the two molecules [79, 110].

Deficiency in S100A4 is associated with a smaller CD5+CD4+ T-cell population, which presents also low intensity of active pY429 CD5 expression on the T cell membrane. A functional consequence of insufficient CD5 in S100A4-deficient mice may be found in the enhanced lymphocyte proliferative capacity observed in S100A4-defficient mice; however, binding assays revealed only a weak interaction between S100A4 and a CD5 peptide containing the CK2-sensitive sequence. Nevertheless, a functional interaction between CD5 and S100A4 may be indirect, possibly involving other CD5-binding partners, and is worthy to be further explored.

6. AIMS OF THIS THESIS

The immunomodulatory function of CD5 in T cell activation suggests that it can play a major role in disease processes such as autoimmunity and cancer. A comprehensive understanding of how CD5 exerts its regulation, including the identification of its signaling partners, is still missing.

The main goal of this thesis is to extend the knowledge of the CD5 function by dissecting the interactions established through the cytoplasmic tail and evaluate how determinant each tyrosine residue is in early and late signaling events.

It was also a long term objective of our line of research to determine the molecular features regulating the translocation of CD5 to the immunological synapse upon T cell-APC interactions. For that purpose, we are evaluating whether the extracellular domain, the intracellular tyrosine residues, or both, are critical for synapse targeting.

Our ultimate goal is to define whether CD5 is a genuine signaling regulator or whether its function may resemble a membrane adapter that could possibly induce different responses depending on the assembled CD5 signalosome.

METHODS

Plasmid constructs

Full-length cDNA of human CD5 was amplified by PCR from a template in the CD5-pGFP-N1 vector kindly provided by G. Bismuth (Institut Cochin, Paris). To make the desired deletion of the ITAM-like motif (YSQPPRNSRLSAYPAL) in the CD5 cytoplasmic domain, or to mutate tyrosine residues into phenylalanines, mutagenic oligonucleotides were used according to the manufacturer's protocol of the QuikChange site-directed mutagenesis kit (Stratagene).

A lentiviral expression vector based on pHR-SIN [111] encoding citrine downstream of the insertion site was used. CD5 wild-type and mutants were cloned into this vector using the *Mlu*I and *Bam*HI restriction sites for constructs with citrine, and using *Mlu*I and *Not*I restriction sites for constructs without citrine. The accuracy of the sequences of if the final constructs was checked by sequencing.

Cell lines

Cell lines Jurkat TAg (JTAg), Raji, E6.1, as well as JTAg cell lines stably transfected with human CD5 mutants were maintained at 37 ºC and 5% CO_2in RPMI 1640 culture media supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin G (50 U/ml) and streptomycin (50 µg/ml). Human embryonic kidney HEK293T cells[112] were grown at 37°C in a 5% CO_2 humidified chamber, in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin G (50 U/ml) and streptomycin (50 µg/ml).

Lentiviral transduction

HEK-293T cells[112] were transiently transfected with pHR-SIN vector constructs, together with pMD.G and p8.91 lentiviral vectors [113] in 6-well plates using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Supernatant was harvested at 72 h after transfection and centrifuged at 1,800x*g* for 5 min at RT to remove any debris from supernatant. $1x10^6$CD5-deficient JTAgcells[114] were platted into a T25 flask with 2 ml of fresh complete RPMI 1640and left to transduce with lentiviral supernatants overnight. Cells were maintained at 37 °C in a 5% CO_2 humidified incubator.

Antibodies

Monoclonal antibodies (mAb) used were: mouse anti-human CD5 Y2-178 (Santa Cruz Biotechnology); mouse anti-human CD3 clone OKT3 (Exbio); mouse anti-human CD28 clone 28.2 (Exbio); mouse anti-human CD6 clone Mem98 (Exbio); rabbit anti-human CD5 RabmAb (Abcam); mouse anti-human CD69 APC-conjugated (eBioscience); mouse anti-human CD3 PerCP-Cyanine5.5 conjugate (eBioscience). Isotype control mouse anti-human C23 (Santa Cruz Biotechnology).Polyclonal antibodies were: rabbit anti-human Crk (Abcam); rabbit anti-human S100A4 (Dako); rabbit anti-human Csk (Santa Cruz Biotechnology); rabbit anti-human Fyn (BL90) and rabbit anti-human Lck (DA3) were given by J. B. Bolen and M. G. Tomlinson (University of Birmingham). Secondary antibodies were: goat anti-rabbit peroxidase conjugate (Sigma); IgG VeriBlot anti-rabbit for immunoprecipitation assay (IP) peroxidase conjugate (Abcam); donkey anti-mouse IgG conjugated with Alexa Fluor 647 (Life Technologies).

Flow cytometry analysis

For the detection of surface receptors, 5 x 10^5cells were washed twice in ice-cold phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) and 0.1 % NaN_3 (PBS/BSA/NaN_3). Cells were incubated with primary antibodies diluted at proper concentrations in PBS/BSA/$NaN_3$and incubated on ice for 30 min. Cells were washed and stained with secondary antibodies, if applicable, for additional 30 min on ice and protected from daylight. At the end, cells were resuspended in PBS/BSA/$NaN_3$and 10,000 live cells were collected on a FACSCalibur (BD Biosciences). The data were analyzed using the FlowJo software (Treestar).

Fluorescence-activated cell sorting

In order to have the different JTAg CD5 mutants expressing equivalent amounts of surface CD5 and CD3 throughout the different cells, 1 x 10^7 cells were centrifuged for 5 min at 300 x *g* and washed twice with PBS/BSA/NaN_3. Cells were stained for CD5 and CD3 surface expression using an IgG1 mouse anti-human CD5 mAb (Y2-178, Santa CruzBiotechnology) followed by a donkey anti-mouse IgG-Alexa Fluor 647 conjugated (Life Technologies) and an IgG2a mouse anti-human CD3 conjugated with PerCP-Cy5.5 (eBioscience). Cells were washed and ressuspended in PBS containing 5 mM ethylenediaminetetraacetic acid (EDTA), 25 mM 4-(2-

hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 2 % FBS and sorted on a FACSAria 1 cell sorter (Becton Dickinson). In some cases, the levels of citrine fluorescence were also taken into account to perform the sorting.

Fluorescence microscopy and analysis of CD5 membrane localization

JTAg cell lines stably transfected with human CD5 mutant cDNAs in frame with citrine fluorescent protein were plated on poly-L-lysine-coated glass coverslips for 30 min at 37°C. Cells were then washed and fixed in 4 % paraformaldehyde (PFA) in PBS at RT for 10 min. Following washing, cells were counterstained with the nuclear dye 4',6-diamidino-2-phenylindole (DAPI; Life Technologies)and mounted on a glass slide. Immunofluorescence and transmission light images were acquired on an Zeiss Axio Imager Z1 fluorescence microscope (Carl Zeiss) equipped with an AxioCam MR 3.0 video camera (Carl Zeiss) and Axiovision 4.7 software (Carl Zeiss, Germany).

Intracellular calcium flux

To measure total intracellular calcium release as a measure of cell activation in JTAg cells expressing the different CD5 mutants, 3 x 10^6 cells were loaded with the calcium indicator Fluo-4 (Molecular Probes, Life Technologies) at the final concentration of 5µM in complete RPMI and incubated for 30 min at 37 ºC with agitation. After this, cells were washed with PBS/BSA/NaN_3 and the basal calcium levels were analyzed by flow cytometry in the FACSCalibur analyzer (BD Biosciences) for 1 min followed by addition of 2 µg/mL of the agonist CD3 mAb OKT3 (Exbio) and sample monitoring for calcium release during 6 min. As positive control, 2 µg/ml ionomycin (Calbiochem) was added to open all calcium channels. Analysis was performed using FlowJo.

Measurement of CD69 expression

JTAg cells expressing CD5 mutants were plated on a 96-well plate at a concentration of 5 x 10^5 cells per 100µl of complete RPMI. Cells in one half of the wells were kept in resting conditions whereas on the other half were activated with 2 µg/ml of OKT3and 5 µg/ml of the CD28 mAb 28.2 (Exbio). 10 µg/ml of phytohemagglutinin (PHA) was used as positive control for T cell activation. Cells were incubated at 37 ºC for 12 h and stained with mouse anti-human CD69 APC-conjugated (eBioscience) for 30 min. After washing, cells were ressuspended in

PBS/BSA/$NaN_3$and data was collected on a FACSCalibur (BD Biosciences). The data were analyzed using FlowJo.

Proliferation assay

The different JTAg cells (untransfected and transfected with different CD5 cDNA mutants) were subjected to serum deprivation for 24h to synchronize the cell cycle. Cells were then stained with carboxyfluorescein succinimidyl ester (CFSE) using a cell proliferation kit (Molecular Probes, Life Technologies) and resuspended in 1 ml of PBS/BSA/NaN_3. 200 µl of the cell suspensions were used to mark the 0 time point and the remaining was left in culture. Samples were analyzed after 24, 48 and 72 h. Cells were stained with 0.5 µg/ml of propidium iodide (PI) (Sigma) and data were collected on a FACSCalibur. The data were analyzed using FlowJo.

Cell conjugation formation

Raji B cells were incubated with a mix of superantigens (staphylococcal enterotoxins SEE, SEA, SEB and SEC3, 200 ng/ml each; Toxin Technologies) and plated on poly-L-lysine-coated glass coverslips for 30 min at 37°C. Jurkat cells were added to these APCs and then incubated at 37°C for 45 min to promote cell-cell interactions. Cells were fixed with 4% PFA in PBS for 10 min and washed several times with PBS before analysis. Cells were washed, counterstained with DAPI and mounted on a glass slide. Immunofluorescence and transmission light images were acquired on a Zeiss Axio Imager Z1 equipped with an AxioCam MR 3.0, and analyzed using the Axiovision 4.7 software.

Cellular activation/Pervanadate treatment and immunoprecipitation of CD5 in Jurkat cells

4 x 10^7 E6.1 Jurkat cells were used per condition. Pervanadate was freshly prepared by mixing sodium orthovanadate 100 mM (Sigma-Aldrich) and H_2O_2 at a final concentration of 0,6%, 10 minutes before addition to cells at a final concentration of 100 µM. Cells were activated for 2 or 10 minutes with pervanadate at 37 °C. In parallel experiments, E6.1 cells were activated with 5 µg/ml OKT3 $F(ab)'_2$ during 2 and 10 min at 37 ºC.

Cells were lysed for 30 min on ice in lysis buffer [10 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 1% (v/v) Triton X-100]. The nuclear pellet was removed by centrifugation at 11,000 x *g* for 10 min at 4 °C, and the

supernatants were precleared by end-over-end rotation with protein A-Sepharose CL-4B beads (Amersham Biosciences) for 30 min at 4 °C. 5 µg of Y-2/178 and 100 µl of 10 % protein A-Sepharose beads were added to the samples and rotated for 90 min at 4 °C. The beads containing the immune complexes were washed three times in 1 ml of lysis buffer

Samples were boiled for 5 min in SDS buffer and run on 11% SDS-PAGE.

In vitro kinase assays and precipitation of phosphorylated CD5 peptides

Between 1 and 3 x 10^7 E6.1 Jurkat cells were lysed and immunoprecipitated as described in the section above. Antibodies used for immunoprecipitation (2 µl anti-sera antibody) and immunoblotting were polyclonal anti-Fyn (BL90), anti-Lck (DA3) and anti-Csk. In the end of the immunoprecipitation, beads containing the immune complexes were washed 3 times in 1 ml of lysis buffer and 2 additional times in kinase assay buffer (25 mM HEPES and 0.1 % detergent). Triton X-100 assay buffer (30 µl) containing 10 mM $MnCl_2$, 1 mM sodium vanadate, 1 mM NaF, and 50 µCi (185 KBq) of [γ-^{32P}]-ATP was added to the immune complexes, and *in vitro* kinase reactions were allowed to occur for 15 min at 25 °C. Reactions were stopped by the addition of 30 µl of 2 % SDS buffer after which the samples were boiled for 5 min. Products were separated on SDS-PAGE gels, and autoradiography of the dried gels was done with BioMax MR films (Kodak).

When indicated, S100A4, or BSA as control, were included in the kinase reaction. Densitometric quantitation of the autoradiographs was done on a GS-800 densitometer (Bio-Rad) using the Quantity One software (Bio-Rad). Densitometric values are expressed in arbitrary units calculated from background non-saturated signals.

A biotinylated peptide containing the rat CD5 ITAM-like sequence (Biot-AASHVDNEYSQPPRNSRLSAYPALE-OH, purchased from New England Peptide) was also included as a Fyn or Lck substrate in the reaction mix at a final concentration of 0.5 µg/µl. For precipitation of the biotin-labeled CD5 peptide, the beads containing the immune complexes were boiled for 5 min in 2% SDS and diluted 8-fold with lysis buffer. After centrifugation, supernatants were recovered and pre-cleared for 30 min with 100 µl protein A-Sepharose beads. The CD5-peptide was recovered using neutravidin beads (Thermo Scientific) and the incorporated [γ-^{32}P]-ATP was measured in a Beckman liquid scintillation counter.

Western-Blot

Proteins were separated by SDS-PAGE under reducing or non-reducing conditions as deemed appropriated and then transferred in a semi-dry iBlot system to a nitrocellulose membrane (Life Technologies). Membranes were blocked in TBS, 0.2 % (v/v), Tween 20 (TBS-T), containing 5 % (w/v) non-fat dried milk, probed with unconjugated primary antibody for 1 hour and revealed with HRP-conjugated goat anti-mouse or goat anti-rabbit IgG (both from Sigma), or IgGVeriBlot anti-rabbit (Abcam) at appropriate dilutions. Membranes were washed thoroughly with TBS-Tween. Immunoblots were developed using ECL (Amersham Biosciences) and exposed to BioMax MR films (Kodak).

RESULTS

DESIGN OF THE CD5 MUTANTS

CD5 is an important inhibitory receptor that down-modulates signaling and thus controls the strength of T cell activation [68]. As CD5 has no intrinsic enzymatic activity, it is believed that its function is mediated by molecular associations it establishes with downstream effectors. A main mechanism of transient molecular interactions in signal transduction propagation involves the coupling of molecules containing SH2 domains that bind to phosphorylated tyrosine residues of the cytoplasmic part of important surface receptors. A main interest is therefore to characterize the molecular interactions established by individual phosphotyrosines of the cytoplasmic tail of CD5, and using mutational studies, to establish the impact of these very localized associations in the cellular function during activation.

Among the four tyrosine residues present in the cytoplasmic tail of CD5, Y429 andY463 are known to be highly phosphorylated upon T cell activation [58, 59]. On the other hand, tandemly arranged ITAM-lookalike tyrosines Y429 and Y441 have been described to be determinant for the inhibitory effect of CD5 in both B and T cells [80, 89]. Our previous unpublished results suggested that deletion of a sequence encompassing the ITAM-like motif also resulted in the abrogation of CD5 recruitment to the IS.

Taking these data into account, we produced a set of constructs to analyze the molecular function of CD5 (Fig. 6): one containing the cDNA coding for full-length CD5 (CD5 WT), one CD5 mutant having the ITAM-like sequence deleted (CD5 ΔITAM), and two constructs with single of Y429F or Y441F substitutions (CD5 Y429F and CD5 Y441F), where tyrosine residues were individually substituted by phenylalanine, a structurally similar amino acid that is however unable of being phosphorylated and to serve as binding site for other proteins. All constructs were inserted into the pHR plasmid to induce protein expression through lentiviral infection.

In addition, we designed two further constructs, one resulting in the complete removal of the cytoplasmic domain (CD5 ΔCyt, to be used as a control non-signaling CD5), and one other where we removed the sequence coding for the extracellular part (CD5 Headless, with the removal of the three SRCR domains) to evaluate whether CD5 was able to be translocated to the immunological synapse without ligand binding. Depending on the projected experiments, constructs (except the CD5

Headless mutant) were designed to both contain or not mCitrine, a monomeric fluorescent YFP variant, fused to the C-terminal part of CD5 (Fig. 8).

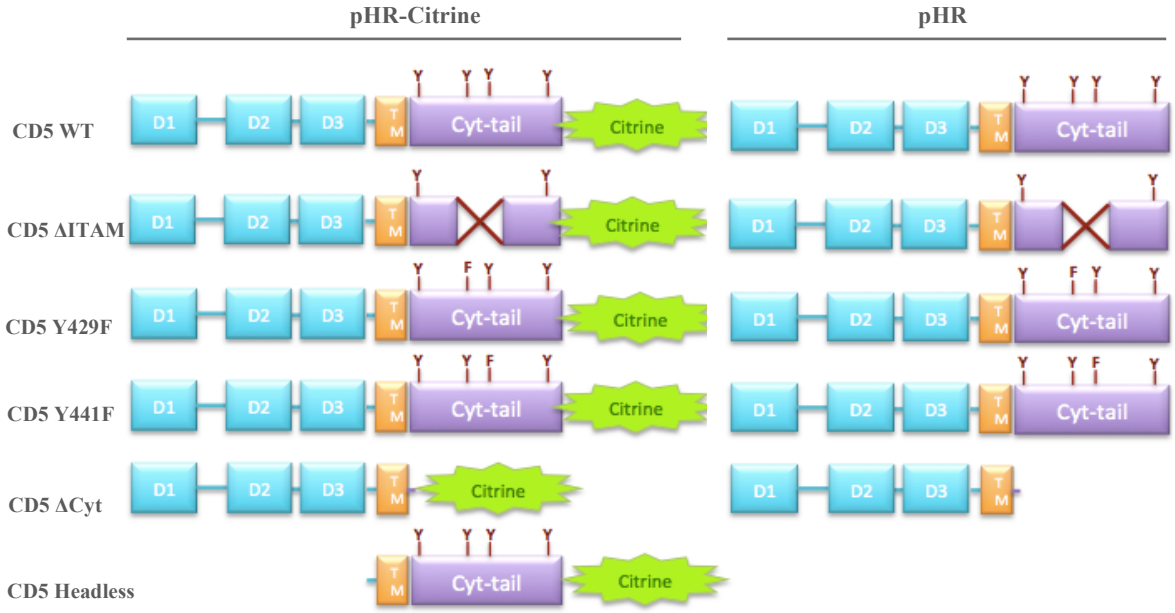

Figure 8. Establishing stable cell lines expressing CD5 mutants. Schematic representation of CD5 wild type (WT) and CD5 mutants: one with the ITAM-like sequence deleted; two constructs where each of the tyrosine residues 429 and 441 were individually substituted by phenylalanine; CD5 ΔCyt without the cytoplasmic tail; and CD5 Headless where the extracellular part was deleted. On the left side there are represented the constructs with mCitrine fused to the C-terminus of CD5 and on the right side the constructs without citrine. Lentiviral mediated delivery of the pHR plasmid was used to drive the expression of these CD5 molecules into CD5 deficient-JTAg cells.

CD5, TCR/CD3 AND CD6 EXPRESSION ON TRANSFECTED JTAG CELLS

E6.1 Jurkat cells are an immortalized T cell leukemia line [115] and probably the most widely used model for in vitro T cell signaling research. JTAg cells derive from E6.1, but they were used in these experiments because they lack constitutive CD5 expression [114].

In order to evaluate the effect of lentiviral infection per se, JTAg cells were transduced with empty pHR vector. These cells were analyzed by flow cytometry and compared with untransduced JTAg cells for the expression of relevant surface markers. During our experiments, we closely monitored the expression of the TCR/CD3 complex, due to its vital role on T cell activation. We also analyzed the expression of CD6 that, like CD5, has a modulatory function on T cell signaling, and that has been reported to inversely change its expression to compensate for CD5 fluctuations.

Our initial analyses indicated that in fact some changes in the expression of, in particular, CD3 could be observed following transduction using the empty pHR vector, reinforcing our need to closely evaluate cell surface receptor variations (Fig. 9).

In light of these variations induced by the pHR vector, cells expressing the CD5 Δ Cyt construct schematized in Figure 8 are perhaps a better "negative" control than untransduced cells, given that CD5 Δ Cyt does not signal but the cells have gone through the same experimental procedure and infection protocol like the cells expressing all the other signaling-competent CD5 molecules.

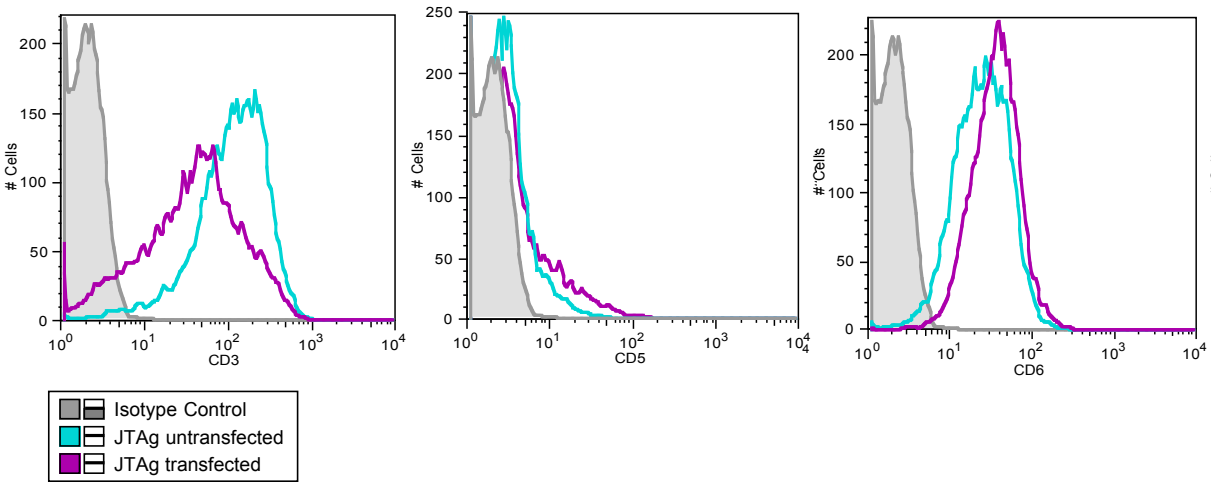

Figure 9. Effect of lentiviral infection on the expression of surface markers (CD3, CD5 and CD6). Lentiviral-mediated delivery of the pHR plasmid induced some variation in the expression of surface markers, most noticeably a detectable decrease of surface TCR/CD3.

JTAg cells were then transduced with all the CD5 constructs using the culture supernatants of 293T cells transfected for 72 h. CD3 expression was analyzed by flow cytometry in all cell lines and, as feared, its expression varied considerably (Fig. 10). Moreover, the expression of the CD5 molecules was also inconsistent, although it was possible to correct the levels of expression using different concentration of virus supernatant during infection.

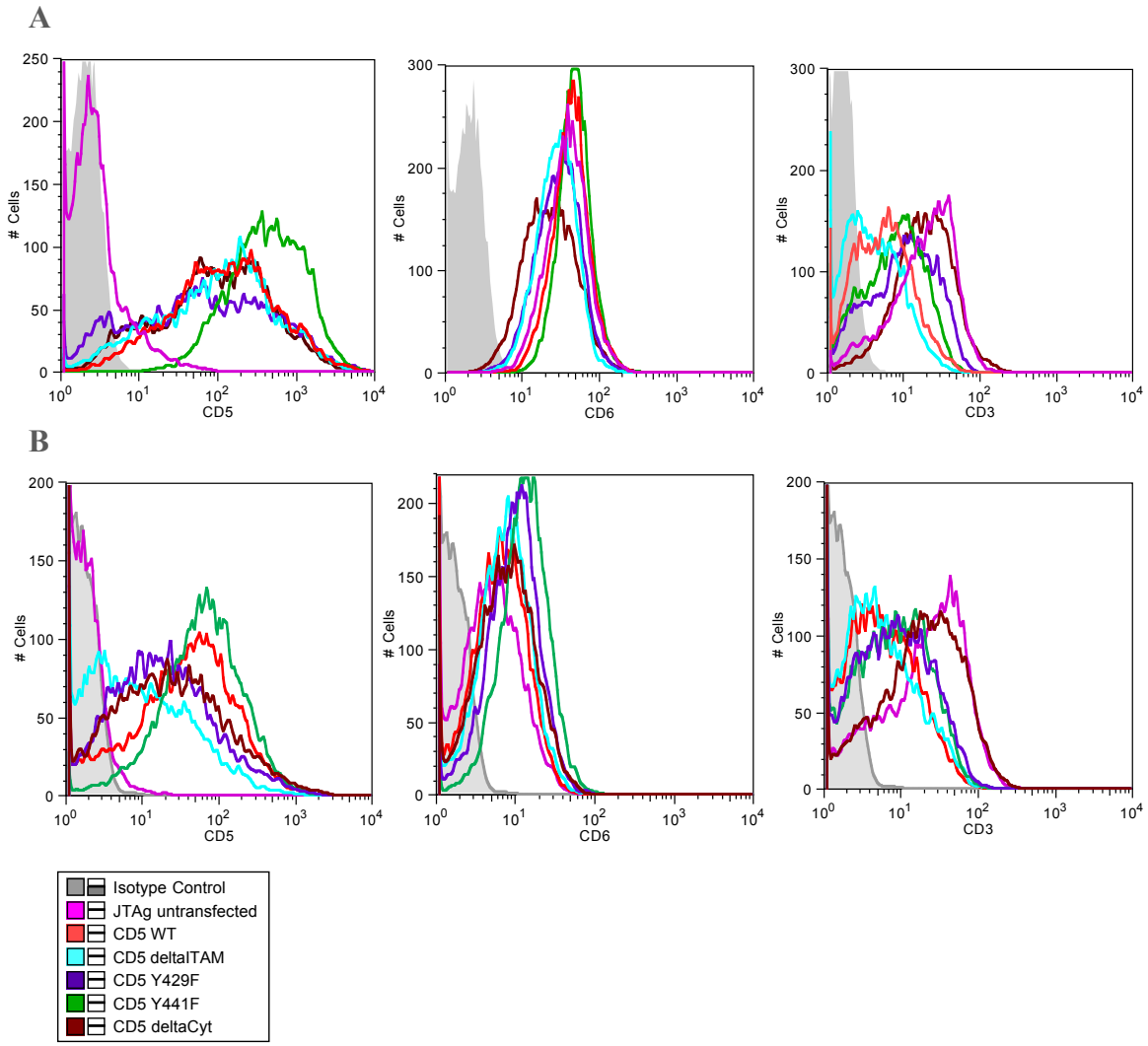

Figure 10. Expressing of relevant cell surface markers in JTAg cells transduced with CD5 variants. Cells expressing wild type CD5 and CD5 mutants without **(A)** or with mCitrine fused to the C-terminus **(B)** were analyzed by flow cytometry. The expression of endogenous CD3 and CD6, and the exogenous CD5 molecules is shown.

In order to obtain all cells with the closest CD3 and mutant-CD5 expression levels, we decided to sort the cells, gating for equivalent patterns of expression. Cells were stained for CD5 and CD3 using respectively Y-2/178 followed by donkey anti-mouse IgG-AlexaFluor 647 conjugated, and mouse anti-human CD3 conjugated with PerCP-Cy5.5, and sorting was obtained using a FACSAria 1 cell sorter. In the case where CD5 is fused with citrine, the citrine fluorescence could also be used to perform the sorting.

After sorting, expression of the surface markers was again evaluated. As shown in Figure 11, there was in general a significant improvement in terms of homogeneity of the expression of CD3 and of the different CD5 mutants, although

some minor variations could still be observed. The expression of CD6 was overall more stable and homogeneous before or after sorting.

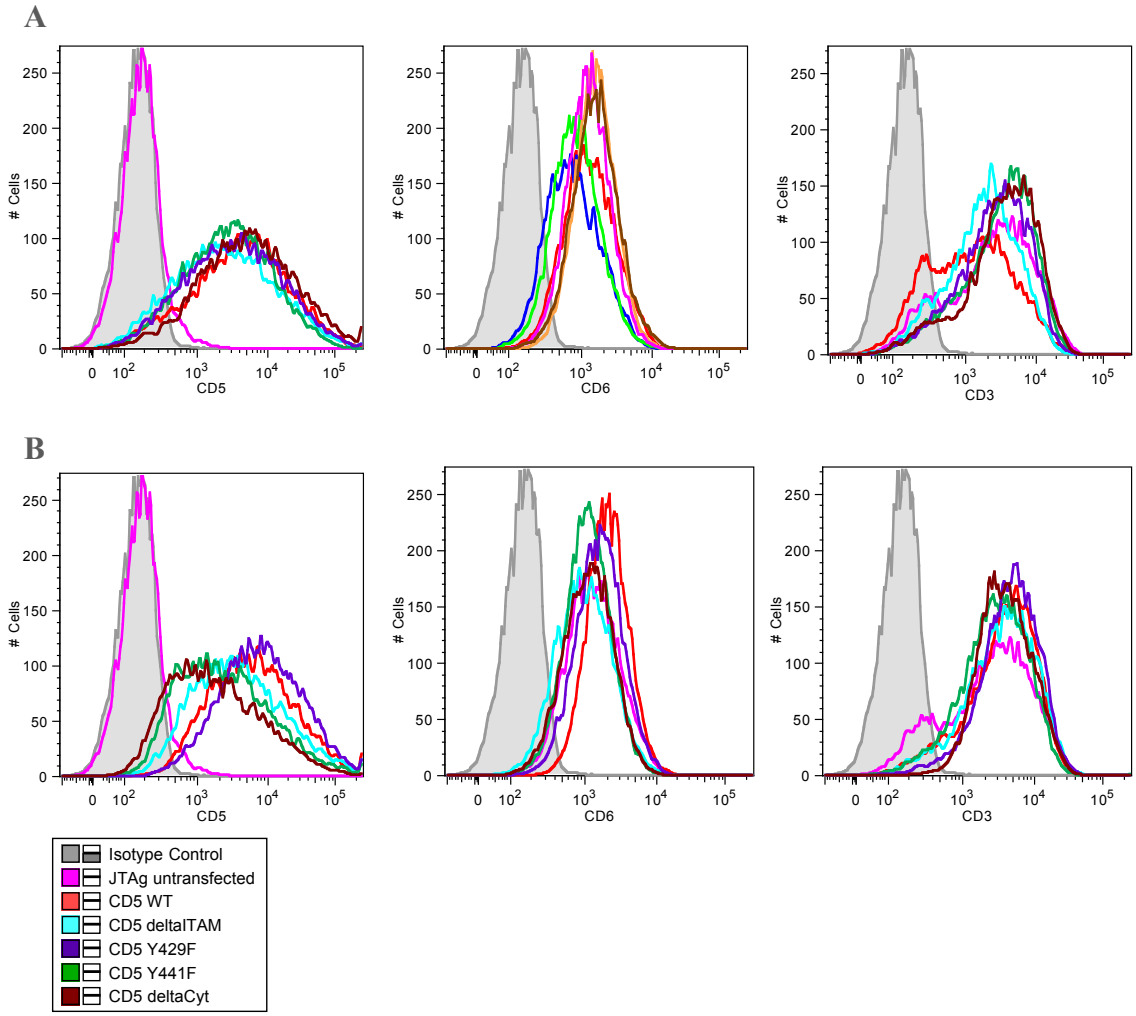

Figure 11. CD3, CD5 and CD6 levels in JTAg-transduced cells after cell sorting. Cells expressing wild type CD5 and CD5 mutants without **(A)** or with mCitrine fused to the C-terminus **(B)** were analyzed by flow cytometry after cell sorting selecting equivalent levels of CD3 and CD5. The expression of endogenous CD3 and CD6, and the exogenous CD5 molecules is shown.

THE CD5 HEADLESS MUTANT IS EXPRESSED AT THE PLASMA MEMBRANE

The CD5 mutant lacking the extracellular domain, which we have called CD5 Headless, was designed with one main goal: to evaluate whether CD5 is able to localize at the immunological synapse in the absence of an APC-expressed CD5-ligand. This mutant only contains 10 amino acids of the extracellular part (corresponding to the linker between the third SRCR domain and the transmembranar sequence) but that are nonetheless required for stable expression,

and together with the transmembrane sequence ensure the attachment to the plasma membrane. Given that the mutant lacks the extracellular part and therefore the binding site for the anti-CD5 mAb used for staining, we could only track its expression by the fluorescence detection of the citrine part of the chimera.

Nevertheless, transduced JTAg cells were analyzed for the expression of CD6 and CD3 using mAbs, before and after CD5-citrine + CD3 sorting (Fig. 12). However, it was not evident whether CD5 Headless was being expressed at the cell surface. To determine the cellular localization of the mutant, we analyzed its expression by fluorescence microscopy. As observed in Figure 13, CD5 Headless can be addressed to the plasma membrane. We could also observe a significant amount of the citrine-derived signal localized near the nucleus (identified by DAPI staining), which could correspond to CD5 Headless molecules being localized at the Golgi complex, where CD5 possibly accumulates before being transported to the cell membrane.

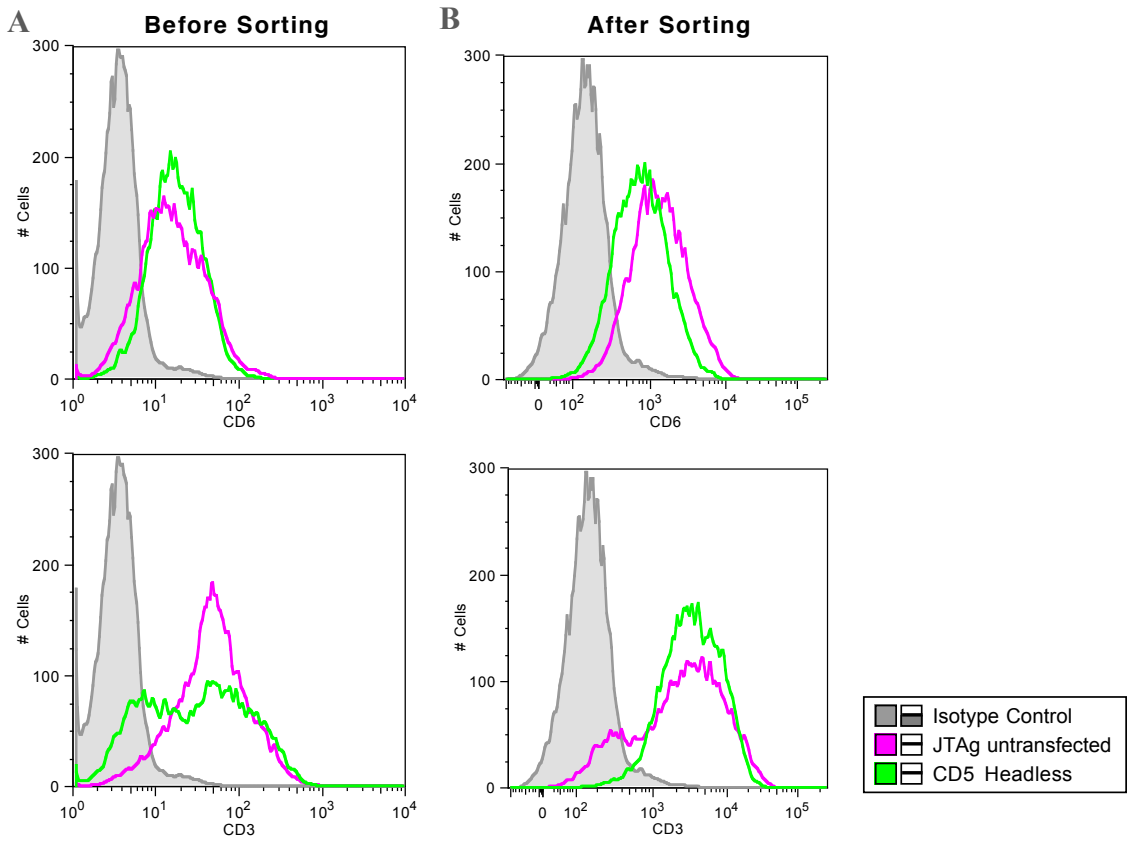

Figure 12.Expression of cell surface markers in CD5 Headless-expressing JTAg cells. Flow cytometry analysis using mAbs against CD6 or CD3 showing the amount of protein expression at the cell surface before **(A)** and after **(B)** sorting. The expression of CD6 was slightly changed but that of CD3 improved significantly regarding homogeneity.

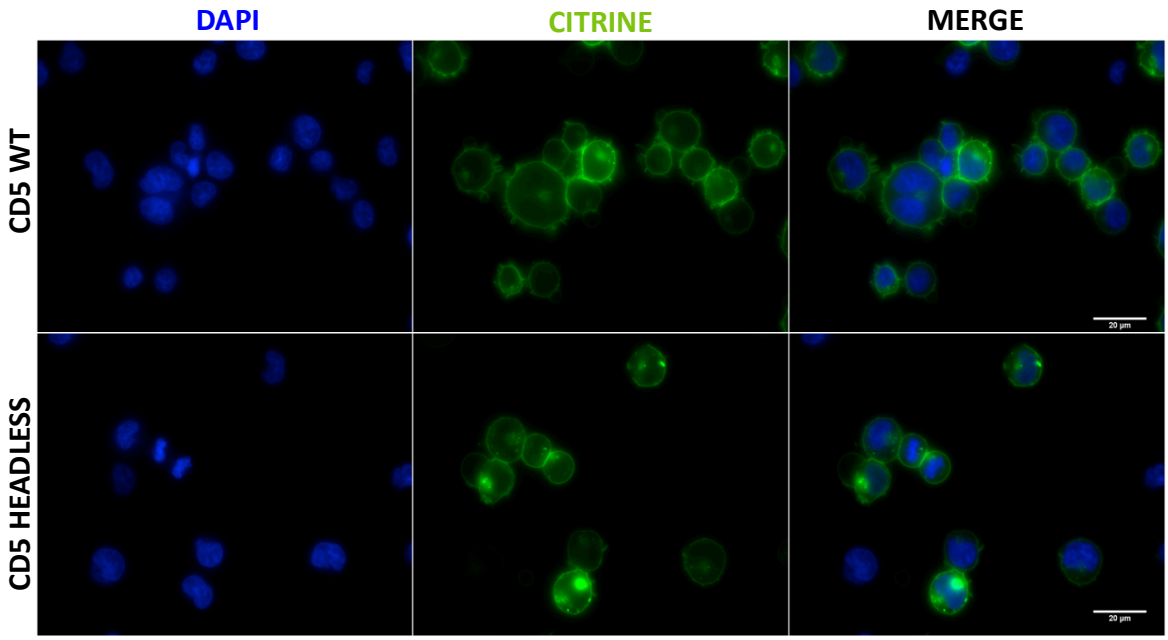

Figure 13. CD5 Headless localizes at the plasma membrane in stably transduced JTAg cells. Cells were transduced with pHR vectors containing constructs encoding wild type CD5 or CD5 devoid of its extracellular domain, both fused to mCitrine at their C-termini. The localization of CD5 WT (upper panels) or CD5 Headless (lower panels) was visualized by fluorescence microscopy showing CD5 molecules in green, in paraformaldehyde fixed cells. Cell nuclei are stained with DAPI and visualized in blue. Scale bar: 20 µm.

The next step in our programmed plan was to incubate these cells with superantigen-loaded Raji cells used as APC, and track the movement of CD5, or lack of it, towards the immunological synapse. These procedures were executed but in the initial experiments we were not able to have the system perfectly adjusted, with no optimal T cell to APC ratios, and therefore no usable results could be collected in due time for presentation in this thesis.

CD5 AS A REGULATORY MOLECULE OF T CELL ACTIVATION

The mechanism of CD5-mediated inhibition has not been fully clarified, but ample evidence suggests that phosphorylation of some of its cytoplasmic tyrosine residues and coupling of downstream inhibitory enzymes are involved. In order to measure the activation status of our cellular models expressing CD5 mutants, and thus to assign specific effects to the mutated tyrosine residues, we performed several assays addressing specific marks of activation: immediate, early and late signaling events, by measuring intracellular calcium influx, CD69 expression and proliferation rates, respectively.

CD5-mediated calcium flux upon T cell activation

Intracellular Ca^{2+} release is an early occurrence in T cell activation, reflecting the initial stages upon TCR triggering, namely the phosphorylation and activation of PLC-γ1 that directly results in phospholipid hydrolysis and calcium mobilization [5]. Free intracellular Ca^{2+} can be detected by Fluo-4, a non-fluorescence compound that when binds to free Ca^{2+} emits fluorescence [116]. In these assays, our CD5-citrine chimeras cannot be used as the emission waves of the two fluorochromes overlap.

JTAg cells expressing the different CD5 mutants were incubated with Fluo-4, and then activated with the addition of OKT3 to the medium. Calcium release was monitored by flow cytometry, with data collection starting 1 min prior to the actual addition of the stimulating antibody (Fig. 14). No differences were observed in the responses between untransfected cells and cells expressing CD5 ΔCyt, as expected, confirming the usefulness of this cytoplasmic tailless CD5 mutant as a negative control (Fig. 14A).

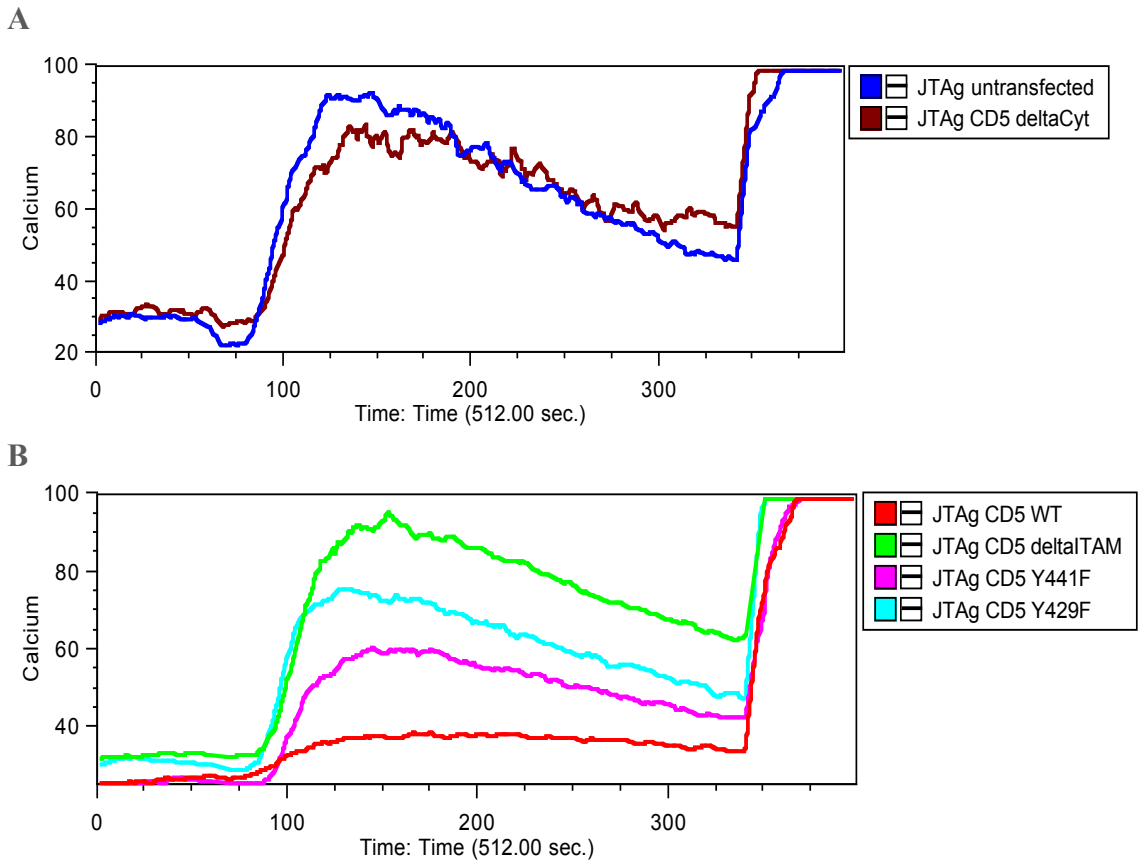

Figure 14. The cytoplasmic tail of CD5 mediates the inhibition of calcium influx upon T cell activation. JTAg cells were incubated with Fluo-4 and analyzed by FACS during 300 sec. Activating CD3 mAb (OKT3) was added at 60 s leading to calcium influx. At the end of each assay, ionomycin was added to fully open calcium channels to give a measure of total cellular calcium. **A.** Levels of intracellular calcium in JTAg CD5 ΔCyt cells comparing to calcium release in untransduced JTAg cells. **B.** Calcium fluxes triggered upon TCR/CD3 stimulation with OKT3 in JTAg cells expressing the different CD5 variants.

Analyzing calcium responses for the cells expressing the different CD5 mutants, it is noticeable that the expression of a complete unmodified CD5 form results in a marked down regulation of calcium signals (Fig. 14 B). On the other hand, the CD5 mutant without the ITAM-like sequence had completely lost the inhibitory capacity, and single substitutions of the tyrosine residues resulted in partial effects, with the Y429 residue being responsible for most of the observed inhibition of calcium signaling (Fig. 14 B).

CD69 expression as a measure of effective T cell activation

CD69 is a lymphoid antigen that is rapidly induced after T cell activation. This molecule is involved in lymphocyte proliferation and other signaling functions, and the increase of its expression is regarded as a measure of a second stage of activation [117].

CD69 levels on JTAg cells expressing the different CD5 mutants were assessed following the simultaneous stimulation of the TCR/CD3 complex (using OKT3) and CD28 (using the 28.2 mAb). An alternative and perhaps stronger stimulus was provided in parallel experiments by PHA-P, a lectin that crosslinks glycosylated surface proteins, including the TCR, and provokes extensive receptor aggregation and cell activation[118].

Cells were activated for 12 h, and then surface CD69 was detected by flow cytometry. Upon 12h of activation with PHA-P, 47.1 % of untransduced JTAg cells expressed CD69 (Fig. 15A), confirming that JTAg cells are “activatable”, whereas less than 10 % of cells were $CD69^+$ following CD3+CD28 stimulation. We nevertheless decided to continue the experiments using mAb-mediated stimulation given that it is more specific to activate certain receptors and that we are looking for a very defined and subtle regulation at the level of surface receptors.

CD69 expression was thus analyzed in JTAg cells expressing the CD5 variants following cell activation using CD3+CD28 mAbs (Fig. 15B), and values were normalized for cells expressing the non-signaling CD5 mutant ΔCyt (Fig. 15C, expressed as fold decrease comparing with JTAg-CD5 ΔCyt). It is apparent that compared with the cells expressing CD5 ΔCyt, all other CD5 mutants failed to induce CD69 expression. In other words, cells expressing CD5 mutants containing the cytoplasmic tail of CD5, either unmodified or mutated, were unable to induce the expression of this marker of activation.

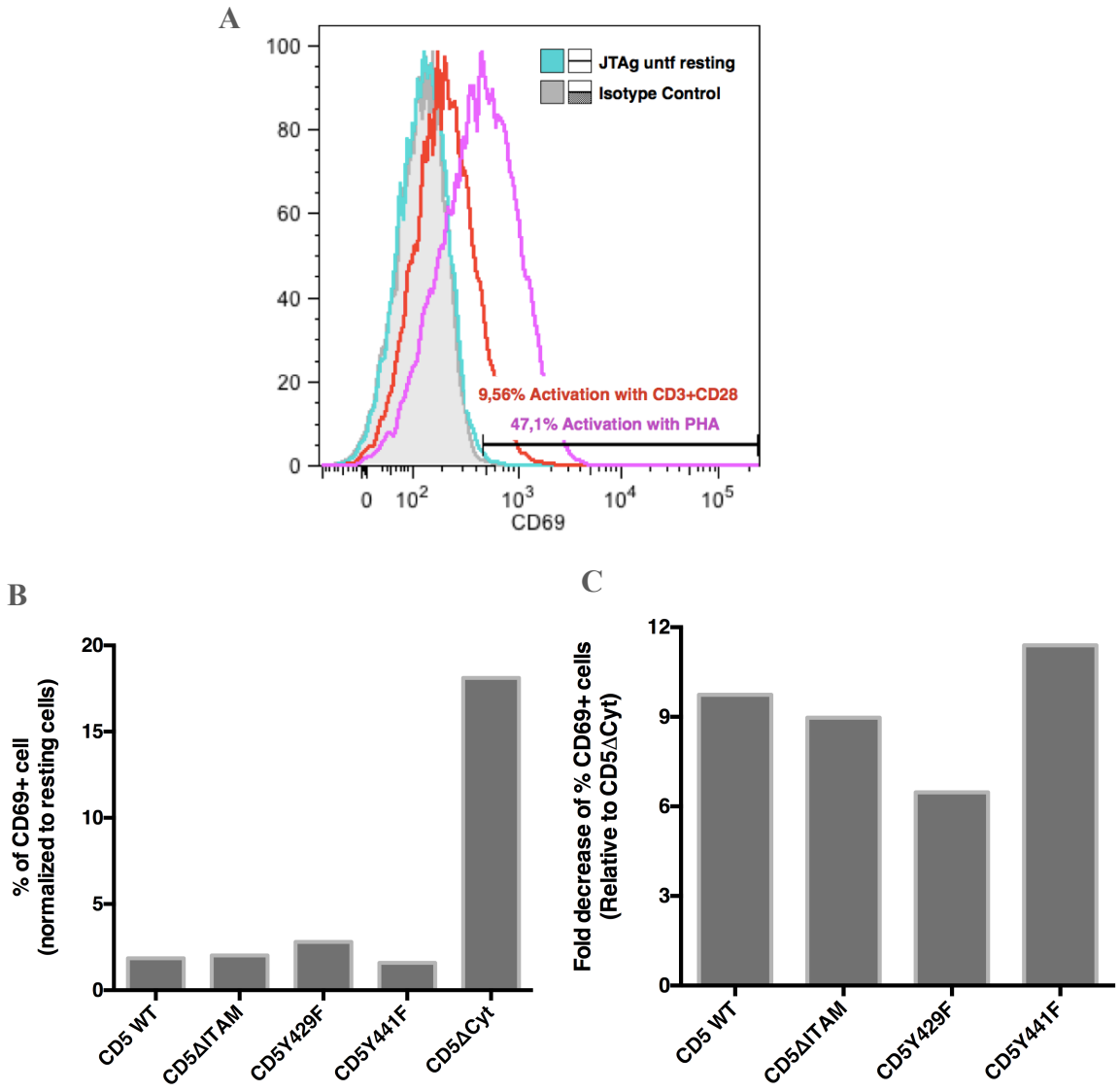

Figure 15. CD69 expression in JTAg cells following cell activation. A. CD69 Expression levels of untransduced JTAg cells activated with 10 µg/ml PHA-P (purple) or with OKT3+CD28 mAbs (red). CD69 expression in non-activated cells (cyan) is negative as it overlaps with irrelevant antibody staining (grey filling). **B**. JTAg cells expressing CD5 WT or mutants were activated with 2 µg/ml OKT3 plus 5 µg/ml CD28, and after 12 h were stained with an anti-CD69 mAb conjugated with APC. Values were normalized to the same population in resting conditions. **C.** Same as in B, with CD69 expression levels in JTAg CD5ΔCyt cells being used as reference to which the values for all the other cell lines are obtained.

The role of CD5 in the inhibition of T cell proliferation

The inhibitory role of CD5 has an impact not only in the early steps of T cell activation but also on cell proliferation. To test the effect of CD5 mutants in the proliferative capacity of JTAg cells, these were serum-deprived for 24h to synchronize cells at the G_1 stage of the cell cycle [119] and labeled with CFSE, which is a fluorescent compound that covalently binds to intracellular molecules and is often used to monitor cell proliferation. Given that when cells divide their components are evenly distributed by the daughter cells there is the perceived halving of intracellular CFSE in each cell cycle [120].

JTAg cells are ever-dividing cells, and to measure the effect of CD5 expression on changes in the proliferation rate, CFSE values were normalized to time 0 (staining and platting of the cells) and to the values obtained with JTAgCD5 ΔCyt cells, as the CD5 ΔCyt mutant is considered signaling-inactive. The CFSE fluorescence levels were analyzed at 24, 48 and 72h, and as shown in Figure 16, JTAg CD5 WT cells had the lowest proliferation rate, as expected. JTAg CD5 ΔITAM and JTAg CD5 Y429F showed proliferation rates close to 1, suggesting that these CD5 mutants had lost the capacity of inhibiting signaling and proliferation. On the other hand, JTAg CD5 Y441F cells had a slight decrease in the proliferation rate, suggesting that mutation of tyrosine residue 441 had less an effect than the Y429F substitution, *i.e.,* Y429 is associated with a slightly higher inhibitory function than Y441.

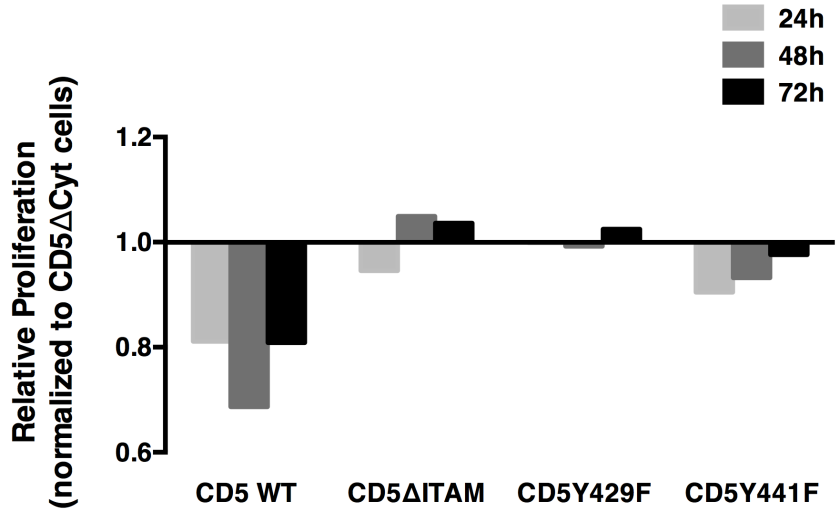

Figure 16. The relative proliferation rate of JTAg cells is influenced by CD5 mutations. After serum deprivation and cell cycle synchronization, non-stimulated JTAg-CD5 cell mutants were labeled with CFSE and their rate of proliferation was analysed after 24, 48 and 72 h by flow cytometry. The results were normalized considering the proliferation rate of the JTAg CD5 ΔCyt cells as 1.

NOVEL BINDING PARTNERS THAT POTENTIALLY MEDIATE CD5 FUNCTIONS

During the last years a number of binding partners for CD5 have been described, that have helped to unveil the mechanism underlying the CD5-mediated inhibitory role. These effectors include enzymes with inhibitory functions such as SHP-1, Cbl and RasGAP, as well as molecules, like PI3 kinase or Fyn, that are sometimes associated with positive signaling but that in the proper context may function to repress activation. We are currently performing, in collaboration with the

group of Simon Davis in Oxford, a wide-range screening of SH2 domain-containing molecules that associate with synthetic peptides that contain the different phosphorylated tyrosine residues of the cytoplasmic tail of CD5. In another direction, we are additionally searching for CD5-binding partners whose interaction with CD5 is not dependent on phosphotyrosine-SH2 interactions.

Crk: a protein adaptader that may relay CD5 signals

During our screening of SH2-containing proteins that interact with CD5, using surface plasmon resonance, we have confirmed a direct interaction of CD5 phosphotyrosines with the SH2 domains of Fyn, PI3 kinase, RasGAP, SHP-1 and Cbl. All these interactions display high affinities of binding of SH2 domains with individual or doubly phosphorylated tyrosines. An additional hit, and in fact one that had one of the strongest affinities, was the SH2 domain of the adapter protein Crk.

In T cells and upon TCR stimulation, Crk participates in a complex that also involves Cbl[93, 121]. However, given that it is an adapter and it is quite difficult to establish which other partners Crk binds to once it docks onto CD5, our strategy will involve in the future to use our CD5 mutants containing Y-to-F substitutions to look for the cellular effects resulting from the mutation of the appropriate tyrosine residues of CD5, as well as from the down regulation of Crk itself. For the time being, we first needed to confirm that in our T cell model Crk does in fact associate with CD5 upon cellular stimulation.

We have used E6.1 Jurkat cells and stimulated them with anti-CD3 (OKT3) antibodies. In these specific activation experiments we have used $F(ab')_2$ fragments of OKT3 because they still contain the two arms including the variable regions and therefore can induce TCR/CD3 dimerization, but lack the F_c portion and thus are not precipitated in the presence of protein A-Sepharose beads. In parallel, we stimulated E6.1 cells with sodium pervanadate, which induces a global inhibition of phosphatase activity, allowing kinases to become active and drive cell activation [122].

After activation with OKT3-F $(ab')_2$ or pervanadate for 2 and 10 min, cells were lysed with detergent and CD5 was immunoprecipitated using the anti-CD5 mAb Y-2/178 and protein A-Sepharose beads. Immune complexes were washed, heated, and loaded onto SDS-PAGE gels. After electrophoresis and western blotting we detected Crk in CD5 immunoprecipitates using an anti-Crk polyclonal antibody, confirming an interaction between CD5 and Crk in Jurkat cells upon cell activation (Fig. 17).

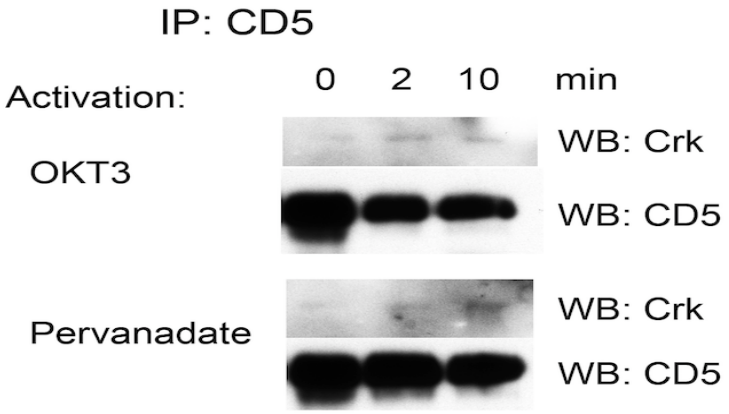

Figure 17. Crk co-precipitates with CD5 in Jurkat cells. CD5 were immunoprecipitated from cell lysates of non-stimulated and stimulatd Jurkat cells. In the upper panel stimulation via TCR/CD3 using OKT3, at timepoint 2 and 10 min. In the lower panel, stimulation with sodium pervanadate for 2 and 10 min. Immunoblot of CD5 is used as a loading control.

S100A4 associates with, and affects the kinase activity of, the Src-tyrosine kinases Lck and Fyn

Lck, and to a lesser extent Fyn, are the protein tyrosine kinases which, concomitantly with the initiation of TCR-mediated signal, are involved in the phosphorylation of CD5 [57]. In collaboration with the group of Maria Bokarewa in Gothenburg, we have established that deficiency of the cytoplasmic calcium binding protein S100A4 results in insufficient CD5 expression and in increased T cell proliferation. We therefore asked whether the S100A4 protein could directly obstruct the activity of the Src-tyrosine kinases towards CD5.

Using polyclonal antibodies, we immunoprecipitated Lck and Fyn from lysates of non-stimulated E6.1 Jurkat cells and tested whether these kinases had their CD5 phosphorylation affected by the presence of exogenous S100A4. Kinase activities were assessed using [γ-^{32}P]-ATP as source of the phosphate groups and as phosphorylation target we used a synthetic biotinylated peptide corresponding to the C-terminal part of the CD5 cytodomain and containing the ITAM-like motif. After the kinase reactions in the presence or different amounts of recombinant S100A4, the CD5 peptide was pulled down with neutravidin beads and the radioactivity incorporated into the peptide was measured in a liquid scintillation counter.

Recombinant S100A4 induced a remarkable decrease of peptide phosphorylation mediated by Lck, while it provoked an increase in the

phosphorylation regulated by Fyn (Fig. 18A). This effect of S100A4 was calcium-independent, since the addition of $CaCl_2$ had only marginal effect of the phosphorylation of the peptides. Replacement of S100A4 by control BSA restored completely (for Fyn) or most (for Lck) the peptide phosphorylation.

To clarify whether these effects were due to changes in the kinases activities by S100A4 or to a possible block/interference of the phosphorylation sites by S100A4 binding to the peptide, we immunoprecipitated the kinases directly from the reactions and assessed their autophosphorylation capacity. As seen by SDS-PAGE and autoradiography, S100A4 at 0.5 mg/ml provoked a marked decrease of the Lck activity (by 55 %), while the activity of Fyn was simultaneously increased by over 30%, as indicated by the densitometry values under each lane (Fig. 18B). Substitution of S100A4 by BSA reverted the activity of the kinases to their original levels.

A direct interaction of S100A4 with both Lck and Fyn was confirmed by co-precipitation of Lck and Fyn with recombinant S100A4 (at 0.1 mg/ml) from lysates of Jurkat cells. Antibodies against S100A4 and Csk were used as positive and negative controls, respectively. Immunoblotting of the immunoprecipitates with an anti-S100A4 antibody confirmed that S100A4 was included in the Lck and Fyn immunoprecipitates (Fig. 18C, upper panel). Reciprocally, Fyn was visible in S100A4 immunoprecipitates (Figure 18C, lower panel). However, no Lck could be detected in S100A4 immune complexes (Fig. 18C, middle panel). Given that recombinant S100A4 is present in large excess relatively to endogenous proteins in this assay, and that in Jurkat cells Fyn is expressed at much lower levels than Lck, these combined results suggest that the interaction of S100A4 with Fyn is significantly stronger than that of S100A4 with Lck.

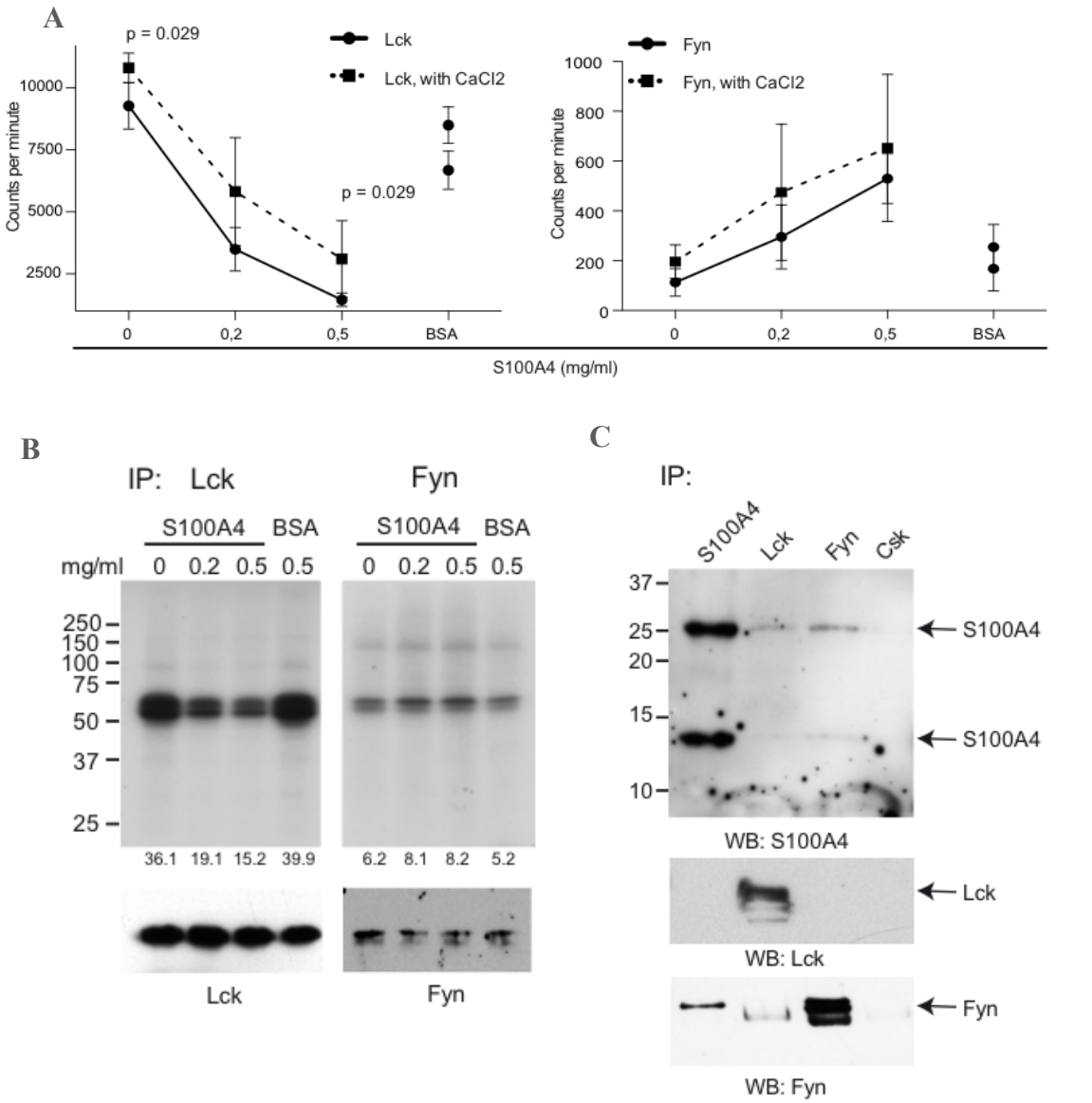

Figure 18. S100A4 co-precipitates with Src-tyrosine kinases Lck and Fyn and changes their kinase activity.A. Lck and Fyn were immunoprecipitated from cell lysates of non-stimulated Jurkat cells. The kinase activities of Lck and Fyn were assessed in the presence or absence of recombinant S100A4 or control mBSA using a 25 amino acid-long synthetic biotinylated peptide corresponding to the rat CD5 sequence and containing tyrosines 453 and 465 of CD5 and [γ-^{32}P]-ATP as source of the phosphate groups. After the kinase reactions, the peptide was pulled down with neutravidin beads and the radioactivity incorporated into the peptide was measured in a liquid scintillation counter. The experiments performed in Ca-free and $CaCl_2$ enriched buffers gave similar results. **B.** Changes in the kinase activity by addition of recombinant S100A4 were assessed by autophosphorylation of kinases. The immunoprecipitated Lck and Fyn kinases were subjected to SDS-PAGE and autoradiography (top panels) and to western blotting using anti-Lck and anti-Fyn antibodies (lower panels, left and right, respectively). Values under the lanes of the top panels represent densitometry of the signals obtained in the autoradiography normalized with the densitometry values obtained from the immunoblots against Lck or Fyn, that are used as loading controls. **C.** Lck and Fyn were immunoprecipitated from lysates of Jurkat cells in the presence of recombinant S100A4 at 0.1 mg/ml. Antibodies against S100A4 and Csk (a tyrosine kinase that phosphorylates the inhibitory C-terminal tyrosine residue of Lck and Fyn) were used as positive and negative controls, respectively. Western blots were performed and indicated the presence of S100A4, Lck or Fyn in the different immunoprecipitates.

DISCUSSION

The CD5 glycoprotein is a T cell surface antigen described to have different roles in immunity, such as regulation of cell death [78, 114, 123], acting as PRR in the recognition of some fungi-wall PAMPs [124], and as a modulator of the T and B lymphocyte activation and differentiation [68, 70, 71]. This role as a regulator of lymphocyte signaling suggests that CD5 may have an underlying role in the development of autoimmunity [125], cancer [114] (relating to the capacity to prevent cell death) and other diseases. In this work we have contributed to unveil and dissect some questions regarding the CD5 inhibitory role in T cell activation.

CD5 contains four tyrosine residues in its cytoplasmic tail; two of these are within in a sequence that resembles the consensus motif for an ITAM [55]. This pseudo-ITAM has, nonetheless, been described to be determinant for the inhibitory effect of CD5 in B and T cells [80, 89] and in our previous unpublished results, we determined that a CD5 mutant without this sequence could not localize at the IS upon antigenic challenge.

To analyze the effect of CD5 tyrosine residues in cell signaling, JTAgCD5-deficient cells were transduced with the CD5-coding cDNAmutants described in Figure 8. The stable cell lines resulting from the transductions express either a full length CD5 form (CD5 WT), a CD5 molecule lacking the ITAM-like sequence (CD5 ΔITAM), two forms where each of the tyrosines were substituted by a phenylalanine, and two other complementaryvariants: one that lackst he complete cytoplasmic tail (CD5 ΔCyt), functioning as a signaling negative control, and another one that is chopped off the extracellular domain. The latter mutant, CD5 Headless, is not capable of establishing extracellular interactions with putative ligands expressed on antigen presenting cells,

The transduction of the cDNAs through the lentiviral system induced unwanted variations in the levels of expression of surface markers like CD6, and more noticeably CD3. However, the control of the expression of surface markers not directly involved in the transfection procedures is critical for the correct setting-up of the system. It was very important to obtain cell lines stably transfected and with similar levels of important receptors like CD3 and CD6, apart form the “inserted” CD5 mutants, to confidently assign variations in the signals obtained to the specific CD5 mutations. To this end, we performed FACS sorting frequently and before the “final” experiments.

A major conclusion from this type of observations in the preliminary experiments was that untransduced JTAg cells were not the proper negative control.

Rather, JTAg cells expressing CD5 ΔCyt, a non-signaling mutant of CD5, were elected as the reference cells as they had experienced all the transformation steps that all other cell lines expressing the CD5 mutants did. In this context, all cell lines have the same background so differences between their responses will be attributable to the CD5 mutations.

Can CD5 localize at the immunological synapse in the absence of an APC-expressed CD5-ligand?

During IS formation, CD5 is one of the molecules that co-localizes with TCR/CD3 complexes and is able to attenuate T cell signaling without damaging IS formation [89]. We have observed that when the extracellular part of CD5 is not able to interact with ligands in the APC, it is still able to translocate to the IS in a cytoplasmic tail dependent manner, relying namely the presence of key motifs in this domain.

The CD5 Headless mutant was designed to evaluate whether CD5 is able to localize at the IS without its extracellular part and consequently without the binding of a putative CD5 ligand expressed in APC or establishing other interactions with molecules expressed on the T cell surface.

Although we have not still completed this line of research, we have established the cell line to be usable in these experiments. As shown in Figure 13, the CD5 Headless mutant is stably expressed and can be addressed to the plasma membrane. The studies to establish the localization of CD5 Headless during APC-antigen presentation are ongoing, with JTAg CD5 Headless cells being stimulated with Raji B cells that present a superantigen in order to establish an IS. The final goal is to perform these assays with video imaging for understanding the kinetics of CD5 Headless recruitment (or not) to the contact zone.

CD5-mediated calcium flux upon T cell activation

Regarding the function of CD5 as a modulator of T cell activation, we have already established the molecular systems to be used, and preliminary performed different assays in order to identify the mechanisms used and molecules involved in the specific stages of activation.

One earliest event is intracellular calcium influx, which corresponds to a very rapid calcium mobilization upon TCR stimulation with the OKT3 monoclonal antibody. In Figure 14A, we could prove that JTAg cells transduced with the

cytoplasmic tailless CD5 mutant can be used as a good negative control, displaying a very similar activation profile compared with the JTAg untransfected cells.

Interestingly, when we delete the ITAM-like motif, the inhibitory pattern observed in the CD5 WT form is completely lost, resulting in the cancellation of inhibition and in the generation of high levels of intracellular calcium. These results confirm the inhibitory role described for the ITAM-like sequence of CD5 [80, 89]. We have gone deeper in the analysis searching for the inhibitory determinants, and have observed that substitution of the Y429 residue by a phenylalanine leads to higher calcium influx compared with the substitution in the Y441 residue, indicating that Y429 is the main responsible for calcium flux inhibition mediated by CD5. However, the calcium signals observed in the JTAg CD5 Y429F cells were not as high as those of the CD5 ΔITAM expressing cells, suggesting that the other tyrosine residue (Y441) might also contribute to signal inhibition, which we in fact did observe (Fig. 14B). We can therefore suggest that both tyrosine residues act in a related and cumulative way to induce full calcium mobilization inhibition.

CD69 expression as a measure of effective T cell activation

The analysis of CD69 expression, which is a surrogate marker of T-cell responsiveness to mitogen and antigen stimulation, is commonly used as a measure of T-lymphocyte activation [126]. In our experiments we evaluated CD69 expression upon activation for 12 h via the TCR/CD3 complex and CD28 (Fig. 15A). However, the level of activation was very low, with less than 10% of the cells having increased CD69 expression. This low reactivity could not be fully accounted by an innate low responsiveness of the cells, as these could be activated to a larger extent with the use of the mitogenic lectin PHA-P. Nevertheless, these values (~50%) are still low, so this protocol should be optimized, perhaps testing different concentrations of the agonists or longer periods of stimulation. For the purpose of our study, we are also planning to use a more physiological and directed mode of activation using superantigen-loaded APC cells, such as Raji cells, which will be recognized by our mutant CD5-expressing Jurkat cells.

Despite the low percentage of CD69 positive cells obtained after activation, we continued with the planned experiments and CD69 levels in activated JTAg CD5-expressing cells were normalized to the values of the corresponding cells in the non-activated state (Fig.15B). Our results again demonstrated that the deletion of the complete cytoplasmic tail (CD5ΔCyt mutant) resulted in CD5 losing its inhibitory

effect and cells become more responsive to stimulation and express higher CD69 levels. However, with all other CD5 mutants, JTAg cells did not increase CD69 expression. This may suggest that the sequences or motifs regulating calcium-dependent signaling and CD69 expression are different in the CD5 molecule and that none of the mutations we used excluded the appropriate signals relating to the control of CD69 expression.

The role of CD5 in the inhibition of T cell proliferation

Proliferation is one of the direct consequences of the T cell activation. Looking to CD5 as an inhibitor of the T cell response, it is expected that the presence of this molecule in the cell surface leads to a decrease intracellular proliferation. Therefore, we investigated the proliferation rate in JTAg CD5 cell lines by staining the cells with CFSE.

In order to have most cells at the same stage of the cell cycle, cells were synchronized by deprivation of serum, which contains growth factors important to the transition between G_0 quiescence and early G_1 [127]. Other methods are available to synchronize cells, such as pharmacological agents or, more physiologically, by contact inhibition and centrifugal elutriation [128].

As observed in Figure 14, JTAg CD5 WT cells proliferate less than the reference JTAg CD5 ΔCyt cells (with values assigned as 1), followed by the mutant expressing theCD5-Y441F substitution. This indicates that, in line wit the results from the calcium assays, the substitution in the Y441 residue results in less inhibition cancellation than the Y429F substitution. On the other hand, the CD5 mutant that lacks the ITAM-like sequence is the one that displays, upon 48 h and 72 h, a more similar response to the reference, as expected given the described role of the ITAM-like sequence.

Taken this together, the calcium and proliferation results are concordant and confirm the inhibitory role of the ITAM sequence, and more precisely, of the Y429 residue, although the other tyrosine may also play a somewhat lesser part.

There are experiments still in line to be performed: to analyze proliferation rates in mAb or mitogenic-activated cells, measure IL-2 production (a cytokine normally secreted by T cells during an immune response to promoted growth, proliferation and differentiation [129, 130]) and evaluate the capacity of CD5-Y429F to translocate to the IS.

Search for binding partners that can relay the CD5 function: Crk and S100A4

Several studies have described the interactions between the cytoplasmic tail of CD5 and different molecules such as PI3K, RAS GAP and c-Cbl [55, 77], SHP-1 [56] and also Lck [27]. We are currently conducting a global phosphotyrosine-SH2 domain screening to uncover novel CD5 mediated interactions. Additionally, we are exploring other directions and studying CD5 interactions not mediated by tyrosine phosphorylation.

In the former class of interactions, using CD5 peptides containing phosphorylated tyrosine residues, and recombinant SH2 domains of over 50 different proteins expressed in Jurkat cells, our surface plasmon resonance studies suggested the possible interaction between CD5 and the adapter protein Crk. Nevertheless, the first step was to confirm that the interaction is held in cellular models, which we did in the current study. The CD5-Crk interaction was however already detected in resting cells, and did not increase significantly upon cell stimulation. This is perhaps one of the flaws of using cell lines that are in permanent cycling and where the activation status is not null. We are confident that repeating these assays using resting and activated primary T lymphocytes, we will be able to see more marked differences, as we have in the past addressing several other activation-dependent interactions.

It is early days to speculate which type of function will Crk bring to CD5, given that as an adapter, Crk bridges many other indirect protein-protein interactions. Crk does interact with Cbl, but so does CD5 directly. Surely we will have more defined ideas once we disrupt the CD5-Crk interaction by mutating the specific tyrosine residue (that would nevertheless unwantedly suppress other CD5 interactions mediated by that tyrosine) or preferentially when we down-regulate the expression of Crk by RNA interference, and then perform a whole battery of experiments testing which signaling functions are altered.

Regarding phosphotyrosine-independent interactions, we unraveled a CD5-S100A4-dependent mechanism of antigen induced T-cell maturation. An S100A4 deficient mouse has increased lymphocyte proliferation, a maturation of T helper cells with low production of IFN-γ and IL17A, low levels of the transcription factor RORγt, which is the most important transcription factor for Th17 differentiation, and shows a Th17 cell malformation and low expression of CD5 and Fyn. Functionally,

the S100A4 knock-out mouse shows also a decrease of morphological signs of arthritis and joint damage. Other results obtained by nuclear magnetic resonance (NMR) indicated a direct interaction between CD5 and S100A4.

More importantly, the fact that the phenotype of the S100A4 knockout mouse shares many similarities with that of the CD5 deficient mice, such as increase in proliferation of lymphocytes and more reactivity to CD3 binding, made us consider to address a possible S100A4 effect on CD5 signaling.

The major function of Fyn in T-cells is the regulation of signaling through TCR where it works in synergy with the Src-tyrosine kinase Lck [131]. Lck and Fyn are known to control TCR signaling through the lymphocyte receptor CD5 (reviewed in [81, 132]). The C-terminal phosphorylation of CD5 by Fyn has been recognized as a mechanism required for CD5-dependent regulation of Fyn [60]. Additionally, CD5 signals are critical for the activation of STAT3 and initiation of Th17 development [110]. We observed, that the deficiency in S100A4 was associated with a smaller $CD5^{+}CD4^{+}$ T-cell population, which had also low levels of the phosphorylated CD5-Y429 in T cells. A functional consequence of insufficient CD5 in S100A4-deficient mice may be found in the enhanced lymphocyte proliferative capacity observed both in S100A4 KO and S100A4-shRNA-treated mice. Analogously to S100A4-deficient mice, functional CD5 is shown to be essential for the differentiation of naïve T cells into Th2 and Th17-cells[79, 110].

The interaction between S100A4 and its target proteins, NMMII, beta-liprin and recently the cytoplasmic domain of FcγRIIIA, occurs within PKC or CK2 sensitive region of the target protein [105, 106, 108, 133]. The cytoplasmic tail of CD5 contains the PKC-sensitive regions at S427 and the CK2 phosphorylation site at its C-terminal region [134]. A deletion of the C-terminal fragment of CD5 containing CK2-phosphorylation site resulted in a failure to produce Th2 and Th17-cell subsets [109]. A binding assay revealed an interaction between S100A4 and a CD5 peptide containing the CK2-sensitive sequence resembling the affinity of smaller myosin peptides [135]. The results of the NMR spectra analysis provided an evidence for the physical interaction between the C-terminal-region of the CD5 cytoplasmic domain and the EF2 sites of S100A4. A dimerization of the CD5 receptor on the cell surface is expected to bring two S100A4 binding sites in each monomer close together and enhance the affinity between CD5 and S100A4.

In *in vitro* experiments we show that S100A4 co-precipitates with both Lck and Fyn from the cell lysates indicating a direct binding between these proteins (Fig.

18). Moreover, this binding had a reciprocal effect on the kinase activity of Fyn and Lck. S100A4 reduced Lck-dependent phosphorylation of CD5 to a degree comparable with that it increased the kinase activity of Fyn. This provides experimental evidence that S100A4 is able to disrupt CD5 complexes at the cell surface that could modify some of the receptor's effects, thus establishing S100A4 as a regulator of CD5 function. Thus, the absence of S100A4 corresponded to highest kinase activity of Lck, which is in agreement with the hyper-proliferation state observed in S100A4-deficient lymphocytes and predicted a disruption of Fyn activity. Insufficient formation of Th17 cells due to reduced activity of STAT3 and poor production of RORγt is consistent with a Fyn-deficient phenotype [136]. These results suggest that S100A4 is essential for controlling a balance between the activities of Lck and Fyn tyrosine kinases. We hypothesized that S100A4 controls Lck-dependent T-cell proliferation and Fyn-dependent differentiation of T helper subsets through a CD5-dependent mechanism.

CONCLUDING REMARKS

We have planned and performed a series of experiments to obtain a deeper knowledge of the mechanisms that confer an immunomodulatory function to CD5. Here we have assigned to the Y429 residue of CD5 a critical role in the inhibition of important signaling pathways that arise from the triggering of the TCR. Our research is still, however, at an early stage and a better understanding of the function of CD5 is expected to be gained when we pursue further this line of research

How exactly CD5 is recruited to the immunological synapse formed between T cells and APCs is still not entirely understood, neither is the eventual dependence on a putative CD5-ligand of the ligand-mediated CD5 translocation. Also, the purpose for the interactions that CD5 establishes with other proteins such as S100A4 and Crk, either depending on phosphorylation or other mechanisms, is still not fully comprehended. Once we gather more mechanistic insights on the molecular function of CD5, we will be able to develop strategies to use CD5 as a therapeutic target to modulate immune responses.

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